METHODS AND MECHANISMS TO IMPROVE ENDOTHELIAL COLONY FORMING CELL (ECFC) SURVIVAL AND PROMOTE ECFC VASCULOGENESIS IN THREE DIMENSIONAL (3D) COLLAGEN MATRICES *IN VITRO* AND *IN VIVO*

Hyojin Kim

Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Department of Biochemistry and Molecular Biology,
Indiana University

August 2015

Accepted by the Graduate Faculty, Indiana University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Mervin C. Yoder, Jr., MD, Chair

David A. Ingram, Jr., MD

Doctoral Committee

Lawrence A. Quilliam, PhD

June 30, 2015

Maureen A. Harrington, PhD

DEDICATION

This thesis is dedicated to my parents, Sunkyum Kim and Hyungsoon Lee, for their endless love, support, and encouragement through my life.

I would also like to thank Jaehoon Yoo, for his love and continued support of my academic endeavors.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Professor Mervin C. Yoder for his mentoring, guidance, and support. I would also like to thank my committee members. Dr. David A. Ingram, Dr. Lawrence A. Quilliam, and Dr. Maureen A. Harrington for their support and feedback during my graduate training.

I would like to thank Momoko Yoshimoto, Michael J. Ferkowicz, Nutan Prasain, Yang Lin, Lan Huang, and Paul J. Crister for their time, training, and intellectual contributions to this work. I would also like to thank Chris Shelly for his support of the laboratory.

Hyojin Kim

METHODS AND MECHANISMS TO IMPROVE ENDOTHELIAL COLONY FORMING CELL (ECFC) SURVIVAL AND PROMOTE ECFC VASCULOGENESIS IN THREE DIMENSIONAL (3D) COLLAGEN MATRICES *IN VITRO* AND *IN VIVO*

Human cord blood (CB) derived circulating endothelial colony forming cells (ECFCs) display a hierarchy of clonogenic proliferative potential and possess *de novo* vessel forming ability upon implantation in immunodeficient mice. Since survival of ECFC post-implantation is a critical variable that limits in vivo vasculogenesis, we tested the hypothesis that activation of Notch signaling or co-implantation of ECFC with human platelet lysate (HPL) would enhance cultured ECFC vasculogenic abilities *in vitro* and *in vivo*. Co-implantation of ECFCs with Notch ligand Delta-like 1 (DL1) expressing OP9 stromal cells (OP9-DL1) decreased apoptosis of ECFC *in vitro* and increased vasculogenesis of ECFC *in vivo*. The co-culture of ECFC with HPL diminished apoptosis of ECFC by altering the expression of pro-survival molecules (pAkt, pBad and Bcl-xL) *in vitro* and increased vasculogenesis of human EC-derived vessels both *in vitro* and *in vivo*. Thus, activation of the Notch pathway by OP9-DL1 stromal cells or co-implantation of ECFC with HPL enhances vasculogenesis and augments blood vessel formation by diminishing apoptosis of the implanted ECFC. The results from this study will provide critical information for the development of a cell

therapy for limb and organ re-vascularization that can be applied to recovery of ischemic tissues in human subjects.

Mervin C. Yoder, Jr., MD, Chair

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

2D ..... Two dimensional

3D ..... Three dimensional

acLDL ..... DiI-labeled acetylated-low density lipoprotein

APC ..... Allophycocyanin

Bad ..... B-cell lymphoma 2-associated death promoter

Bax/Bak ..... Bcl-2-associated X protein

Bcl-2 ..... B-cell lymphoma 2

Bcl-xL ..... B-cell lymphoma extra large

BSA ..... Bovine serum albumin

CB ..... Cord blood

CFU-Hill ..... Colony forming unit-Hill

CNS ..... Central nervous system

Cyt-C ..... Cytochrome c

DAPI ..... 4', 6-diamidino-2-phenylindole dihydrochloride

DISC ..... Death-inducing signaling complex

DKO ..... Double knockout

Dll1 ..... Delta-like 1

E ..... Embryonic day

ECFC(s) ..... Endothelial colony forming cells

ECM ..... Extracellular matrix

EC(s) ..... Endothelial cells

EDTA ..... Ethylenediaminetetraacetic acid

eNOS ..... Endothelial nitric oxide synthase

EPCs.....Endothelial progenitor cells

FACS .....Fluorescence activated cell sorting

FBS..... Fetal bovine serum

FGF..... Fibroblast growth factor

FGFR ..... Fibroblast growth factor receptor

FITC..... Fluorescein isothiocyanate

FLICA.....Fluorescein-labeled inhibitors of caspases

FMO.....Fluorescence minus one

H&E ..... Hematoxylin and eosin

HAECs .....Human arterial endothelial cells

HCP .....Human cord plasma

hESCs.....Human embryonic stem cells

HGF .....Hepatocyte growth factor

HIAECs ..... Human iliac artery endothelial cells

HIF ..... Hypoxia-inducible factor

hMAPCs..... Human multipotent adult progenitor cells

HPL..... Human platelet lysate

HPP ..... Highest proliferative potential

hrbFGF .....Human recombinant basic fibroblast growth factor

hrEGF .....Human recombinant epidermal growth factor

HUVECs .....Human umbilical vein endothelial cells

IL6..... Interleukin 6

LPP ..... Low proliferative potential

MNCs ..... Mononuclear cells

MT1-MMP ..... Membrane type 1-metalloproteinase

NF-κB ..... Nuclear factor kappa-light-chain-enhancer of activated B cells

NO ..... Nitric oxide

NOD/SCID mice ..... Non-obese diabetic/severe combined immunodeficiency mice

OP9-DL1 ..... OP9-Delta-like 1 stromal cells

PBS ..... Phosphate-buffered saline

PDGF ..... Platelet-derived growth factor

PE ..... Phycoerythrin

PE-Cy7 ..... Phycoerythrin-cyanine7

PECAM-I/CD31 ..... Platelet endothelial cell adhesion molecule-I

PI ..... Propidium iodide

PIGF ..... Placenta growth factor

PMPs ..... Platelet microparticles

rhVEGF-A /$VEGF_{165}$ ... Human recombinant vascular endothelial growth factor 165

RT ..... Room temperature

SCF ..... Stem cell factor

SDF-1 ..... Stromal-derived growth factor 1

SDS-PAGE ..... Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SMCs ..... Smooth muscle cells

SRM ..... Serum reduced medium

UCB ..... Umbilical cord blood

UEA-1 ..... Ulex europaeus agglutinin 1

VE-Cad ..... Vascular endothelial-cadherin

VEGF .....Vascular endothelial growth factor

VEGFR1/Flt-1 ..... Vascular endothelial growth factor receptor 1

VEGFR2/Flk-1 ..... Vascular endothelial growth factor receptor 2

αSMA.....Smooth muscle alpha actin

CHAPTER 1. INTRODUCTION

1.1. Development Of Blood Vessels

During organogenesis, the development of the vascular system is one of the earliest events for the proper formation of vertebrate embryos and the system must remain responsive to growth and regeneration signals for the survival of adult tissues and organs (Hannan & Little, 1998; Jin, Beis, Mitchell, Chen, & Stainier, 2005; Lamalice, Le Boeuf, & Huot, 2007; Risau & Flamme, 1995; Wilting & Christ, 1996). Blood vessels deliver oxygen and nutrients, carry away metabolic waste products and serve as the principle interface for the circulating immune cell response to microbes that have invaded tissues (Adams & Alitalo, 2007). The vascular system, composed of functional arteries, veins, and capillaries, originates via two fundamental processes: vasculogenesis and angiogenesis (Figure 1.1.) (Adams & Alitalo, 2007; Lamalice et al., 2007; Risau, 1997).

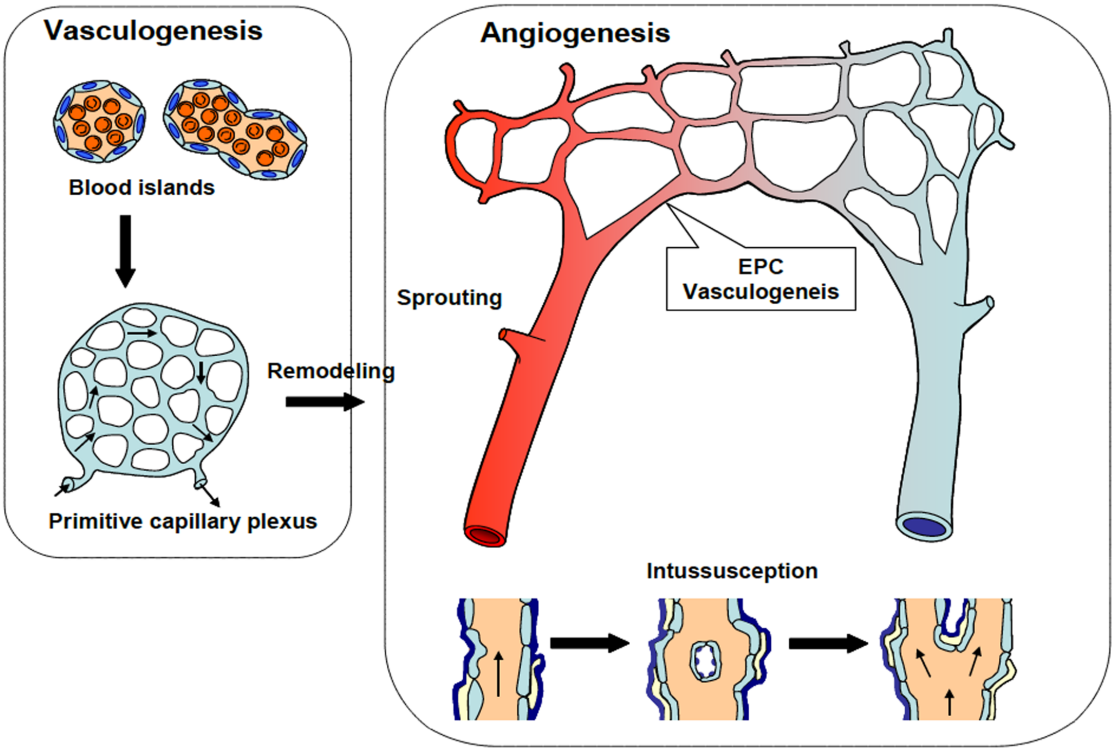


Figure 1.1. A schematic diagram of vascular development during embryonic development. During embryogenesis, angioblasts are derived from mesoderm. Angioblasts give rise to the primary capillary plexus via vasculogenesis before circulation is established. Subsequently, the primary capillary plexus is quickly expanded, recruits mural cells and is remodeled into primary vessels. Primary vessels may be further remodeled through sprouting and intussusceptive angiogenesis and ultimately this leads to the formation of a well-organized arterial, capillary, and venous bed. Reprinted with permission from “Molecular regulation of angiogenesis and lymphangiogenesis” by Adams et al., 2007, Nat Rev Mol Cell Biol, 8:464-478 (Adams & Alitalo, 2007).

Vasculogenesis

During early embryogenesis, on mouse embryonic day (E) 6.5, gastrulation begins with the formation of the primitive streak and leads to formation of three principle germ layers: ectoderm, mesoderm, and endoderm (Hannan & Little, 1998; Risau & Flamme, 1995; Wilting & Christ, 1996; Zorn & Wells, 2009). The first step of blood vessel formation is the differentiation of vascular endothelial cells (ECs), which cover the entire inner surface of all blood vessels. A subset of the primitive mesodermal cells is committed to differentiate into not only the first ECs, called angioblasts but also into hemangioblastic cells in the yolk sac (Adams & Alitalo, 2007; Carmeliet, 2000; Coultas, Chawengsaksophak, & Rossant, 2005; Lamalice et al., 2007; Risau, 1997). The angioblasts migrate and aggregate to form blood islands, which are the earliest discernible vascular structures that give rise to a primitive vascular network in the yolk sac (Adams & Alitalo, 2007; Carmeliet, 2000; Carmeliet et al., 1996; Coultas et al., 2005; Ferrara, 1999; Ferrara et al., 1996; Hannan & Little, 1998; Lamalice et al., 2007; Risau, 1997; Risau & Flamme, 1995; Shalaby et al., 1997; Wilting & Christ, 1996). Fusion of blood islands and subsequent endothelial cell differentiation lead to formation of primitive capillary structures driven by certain growth factors binding to their EC surface receptors (Adams & Alitalo, 2007; Carmeliet, 2000; Carmeliet et al., 1996; Coultas et al., 2005; Ferrara, 1999; Ferrara et al., 1996; Lamalice et al., 2007; Risau, 1997; Shalaby et al., 1997).

EC migration and fusion of the blood islands leads to remodeling into tubular structures that give rise to the first primitive vascular plexus (Adams & Alitalo, 2007; Carmeliet, 2000; Coultas et al., 2005; Lamalice et al., 2007; Risau, 1997). These vascular plexuses remodel into a network of interconnected tubules that provide perfusion of tissues (Adams & Alitalo, 2007; Carmeliet, 2000; Coultas et al., 2005; Lamalice et al., 2007; Risau, 1997). The process of EC lumen and tubule formation (morphogenesis) is critical to establish blood flow and stabilize vessel networks during vascular development (Aplin, Fogel, Zorzi, & Nicosia, 2008; Davis, Koh, & Stratman, 2007; Davis & Senger, 2005; Holderfield & Hughes, 2008; Koh, Stratman, Sacharidou, & Davis, 2008). During capillary morphogenesis, individual ECs generate vesicles after endocytosis (intracellular vacuolation) and interconnect with neighboring cells (fusion) to form multicellular lumens and tubes (Figure 1.2.) (Bayless & Davis, 2002, 2003; Bayless, Salazar, & Davis, 2000; Davis et al., 2007). The intracellular vacuolation process may be observed in ECs without contact with neighbor ECs in 3D collagen matrices (Davis, Bayless, & Mavila, 2002). Many studies have investigated the cellular mechanisms of EC lumen formation in 3D collagen matrices *in vitro* (Davis & Camarillo, 1996; Ilan, Mahooti, & Madri, 1998; Kanzawa, Endo, & Shioya, 1993; Koh, Mahan, & Davis, 2008; Korff & Augustin, 1999; Matsumura, Wolff, & Petzelbauer, 1997; Ment, Stewart, Scaramuzzino, & Madri, 1997; Ng, Helm, & Swartz, 2004; Salazar, Bell, & Davis, 1999; Schechner et al., 2000; Sieminski, Hebbel, & Gooch, 2004; Vernon & Sage, 1999; S. Yang, Graham, Kahn, Schwartz, & Gerritsen, 1999). These studies have reported that matrix-integrin-

cytoskeleton signaling plays a critical role in EC tube formation (Davis & Camarillo, 1996; Ilan et al., 1998; Koh, Mahan, et al., 2008; S. Yang et al., 1999). In response to α2β1 integrin and extracellular matrix (ECM) interactions, Rho GTPase family members Rac1 and Cdc42 are activated to initiate vacuole formation by embedded cells in 3D collagen matrices (Bayless & Davis, 2003; Bayless et al., 2000; Davis & Camarillo, 1996; Koh, Mahan, et al., 2008). After formation of multicellular structures, ECs remodel the surrounding matrix to create vascular guidance tunnels through membrane type 1-metalloproteinase (MT1-MMP) dependent proteolysis in 3D collagen matrices (Stratman et al., 2009). The tunnels allow EC migration throughout the networks and by recruiting pericytes to the newly formed vascular networks, and the new vessels become stabilized. Pericytes stimulate ECs to deposit basement membrane matrices containing collagen type IV, laminins, and fibronectin (Davis & Senger, 2005; Y. Liu & Senger, 2004; Stratman et al., 2009). The deposition of basement membrane matrices contributes to the development of vascular tube maturation, stabilization, and EC quiescence (Davis & Senger, 2005; Y. Liu & Senger, 2004; Stratman et al., 2009).

Growth factors in vasculogenesis

The fibroblast growth factor (FGF) family is composed of 22 FGF ligands that bind to four tyrosine kinases receptors (FGFRs) and share a conserved sequence of 120 amino acids (Ornitz & Itoh, 2001). FGF has been reported to be necessary for the differentiation of mesoderm (Amaya, Musci, & Kirschner, 1991;

Cornell & Kimelman, 1994a, 1994b; Feldman, Poueymirou, Papaioannou, DeChiara, & Goldfarb, 1995; Flamme & Risau, 1992; LaBonne & Whitman, 1994; Pajusola et al., 1994; Slack, Darlington, Heath, & Godsave, 1987). Expression of dominant negative mutants of FGF receptor (FGFR) in early Xenopus embryos abolishes wild-type receptor function and fails to induce mesoderm in response to FGF (Amaya et al., 1991). The embryos display defects in gastrulation and posterior development. Overexpression of a wild-type receptor could rescue these defects (Amaya et al., 1991). FGFR1 deficient mice die at E9.5-E12 and show defects in cell migration through the primitive streak (Deng et al., 1994; Yamaguchi, Harpal, Henkemeyer, & Rossant, 1994). Adenovirus-driven dominant-negative FGFR1 expression results in significant defects in blood vessel development and maintenance in mouse embryos cultured *in vitro* (S. H. Lee, Schloss, & Swain, 2000). $FGFR2^{-/-}$ embryos die at E10.5 because of defects in the placenta and limb buds (De Moerlooze et al., 2000; Eswarakumar et al., 2002; Xu et al., 1998). Cultured FGF-4 null murine embryos display severely impaired proliferation of the inner cell mass, whereas growth and differentiation of the inner cell mass is rescued in the presence of FGF-4 protein (Feldman et al., 1995). These reports suggest that the FGF/FGF-receptor system may be a crucial signal transduction pathway for mesoderm induction *in vivo*. However, $FGF2^{-/-}$ mice and $FGF1^{-/-}/FGF2^{-/-}$ double knockout (DKO) mice display normal vascularization (Miller, Ortega, Bashayan, Basch, & Basilico, 2000; Tobe et al., 1998; Zhou et al., 1998). Analysis of mice lacking individual FGF family members revealed a wide range of phenotypes from early embryonic lethality to very mild

defects. These observations may reflect the extensive redundancy of FGF family members and the contribution of other growth factors including vascular endothelial growth factor (VEGF) to vascular development.

The VEGF family belongs to the platelet-derived growth factor (PDGF) family and consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor (PIGF) (Achen et al., 1998; Ferrara, 1996; Jeltsch et al., 1997; Olofsson et al., 1996; Petrova, Makinen, & Alitalo, 1999). Alterative splicing of human VEGF-A mRNA gives rise to five different isoforms of 121, 145, 165 (the most abundant form), 189, and 206 amino acid residues (Ferrara, 1996). In this manuscript, "VEGF" stands for VEGF-A. VEGF binds to two distinct receptor tyrosine kinases, VEGF receptor 1 (VEGFR1 or Flt-1) and VEGF receptor 2 (VEGFR2 or Flk-1) (Neufeld, Cohen, Gengrinovitch, & Poltorak, 1999; Petrova et al., 1999).

Several studies have reported cross talk between FGF2 and VEGF family members during vasculogenesis (Auguste et al., 2001; Gabler, Plath-Gabler, Killian, Berisha, & Schams, 2004; Kanda, Miyata, & Kanetake, 2004; Seghezzi et al., 1998). Expression of dominant-negative FGFR1 or FGFR2 in glioma cells causes a decrease in tumor vascularization by downregulating VEGF expression (Auguste et al., 2001). While FGF2 modulates VEGF expression in ECs (Seghezzi et al., 1998), anti-VEGF-A neutralizing antibodies reduce FGF2-induced vasculogenesis (Seghezzi et al., 1998). Flt-1 blocking antibodies or expression of a dominant negative Flt-1 leads to a significant reduction of FGF2-

induced EC extensions and capillary morphogenesis (Kanda et al., 2004). Thus, pro-angiogenic effects of FGF2 are stimulated by VEGF/VEGFR signaling in ECs (Gabler et al., 2004).

VEGF/VEGFR signaling is essential for embryonic vascular development (Carmeliet, 2000). In the presence of VEGF, embryonic stem cell-derived endothelial structures generated *in vitro* lead to improved survival of ECs and to the formation of primitive endothelial tubes, whereas FGF2 enhances only survival of angioblasts (Kazemi et al., 2002). In embryoid bodies in which VEGF/VEGFR function is blocked, FGF2 stimulation promotes formation of EC clusters, but fails to stimulate EC development into primitive vessels (Magnusson et al., 2004). In contrast, in $FGFR1^{-/-}$ embryoid bodies, VEGF stimulates formation of a vascular plexus (Magnusson et al., 2004). Thus, FGF2 is a downstream target of VEGF signaling and FGF2 and VEGF may have differential roles in vasculogenesis.

Deletion of a single VEGF allele ($VEGF^{+/-}$) or both alleles ($VEGF^{-/-}$) is embryonic lethal (E8.5) as a result of failure to form the primary capillary plexus (Carmeliet et al., 1996; Ferrara et al., 1996). In addition, VEGF receptors (Flt-1 and Flk-1) play a critical role in vascular development. Flk-1 is expressed on angioblasts and ECs in avian and murine embryos and plays a critical role in differentiation of mesoderm to endothelial and hematopoietic lineages in early development (Dumont et al., 1995; Eichmann, Marcelle, Breant, & Le Douarin, 1993; Flamme,

Breier, & Risau, 1995; Fong, Rossant, Gertsenstein, & Breitman, 1995; Millauer et al., 1993; Roman & Weinstein, 2000; Yamaguchi, Dumont, Conlon, Breitman, & Rossant, 1993). Flk-1 deficient mice (Flk-$1^{-/-}$) display defects in blood island formation and development of hematopoietic and ECs at E7.5 (Shalaby et al., 1995). The Flk-1 deficient embryos die by E8.5 (Shalaby et al., 1997; Shalaby et al., 1995). VEGF receptor 1 (Flt-1) deficient mice display abnormal vasculature and embryonic death marked by disruption of endothelial cell assembly and loss of blood island integrity (Breier, Albrecht, Sterrer, & Risau, 1992; de Vries et al., 1992; Fong et al., 1995; Millauer, Shawver, Plate, Risau, & Ullrich, 1994; Millauer et al., 1993; Shalaby et al., 1995). Thus, VEGF signaling is required for vascular development.

Angiogenesis

After the primary vascular plexus is formed by vasculogenesis, blood vessels are expanded and remodeled rapidly through generating more ECs that form new capillaries by sprouting or splitting from the existing vessels (intussusceptive angiogenesis) (Fig. 1.2.) (Adams & Alitalo, 2007; Carmeliet, 2000; Carmeliet et al., 1996; Coultas et al., 2005; Ferrara, 1999; Ferrara et al., 1996; Lamalice et al., 2007; Risau, 1997; Shalaby et al., 1997). This process, referred to as angiogenesis, is defined as the formation of new vessels from the pre-existing primary plexus. In many regions such as the yolk sac, central nervous system (CNS), and retina, angiogenic sprouting occurs and is characterized by leading EC tip cells and trailing stalk cells controlled by Notch signaling (Fig. 1.2./1.3.)

(Breier & Risau, 1996; Gerhardt et al., 2003; Kurz, Gartner, Eggli, & Christ, 1996; Noguera-Troise et al., 2006; Ridgway et al., 2006).

During sprouting angiogenesis, tip cells lead the way in a branching vessel and stalk cells elongate the sprout to form lumenized tubular structures (Fig. 1.3.) (Gerhardt et al., 2003; Kume, 2009). ECs stimulated by VEGF compete for tip cell position via Dll4/Notch signaling. In tip cells, Dll4 expression is induced more than in neighboring stalk cells to suppress the same response in adjacent ECs via activated Notch signaling (Hellstrom et al., 2007; Leslie et al., 2007; Siekmann & Lawson, 2007; Suchting et al., 2007). Dll4 expression is induced in response to VEGF/Flk-1 signaling in tip cells whereas Dll4 expression activates Notch signaling in adjacent cells to reduce the expression of Flk-1 to suppress the tip cell phenotype (Lobov et al., 2007; Noguera-Troise et al., 2006; Sainson et al., 2005; Siekmann & Lawson, 2007; Suchting et al., 2007). Thus, the tip cell phenotype suppression enhances the stalk cell phenotype. Jagged1 also enhances angiogenesis and antagonizes the effects of Dll4-mediated Notch signaling in stalk cells during sprouting angiogenesis (Benedito et al., 2009). Jagged1 loss of function mutants in the retina decreases sprouting while Jagged1 overexpression enhances angiogenesis and increases the number of tip cells (Benedito et al., 2009). Therefore, Dll4 expression corresponds to elevated VEGF-mediated signaling in tip cells, whereas Notch activation suppresses pro-angiogenic response in their neighboring stalk cells.

Angiogenic sprouting of ECs from existing blood vessels is not the only mechanism that contributes to vascular growth. Intussusception (Figure 1.1.) is a separate mechanism that leads to local expansion of blood vessels. This process is the splitting of vessels through the insertion of tissue pillars (Djonov & Makanya, 2005). Little is known about the process but the process is proposed to involve EC proliferation, cell movement, ECM degradation, and the deposition of new ECM molecules (Djonov & Makanya, 2005).

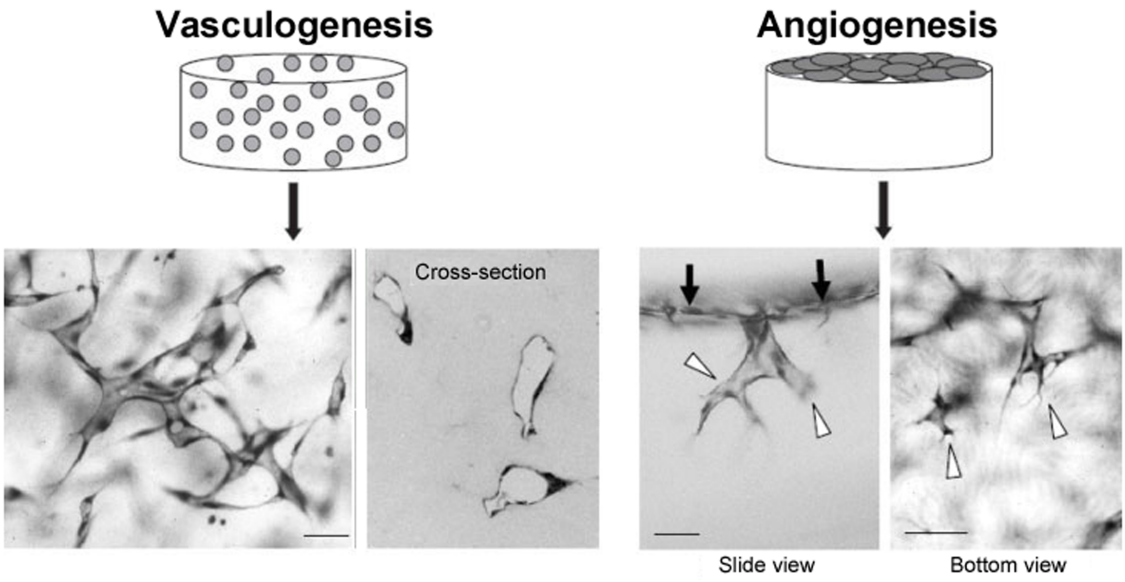


Figure 1.2. Human EC tube morphogenesis in 3D collagen matrices *in vitro*. Vasculogenesis (left panels); ECs were suspended in 3D collagen matrices and EC formed vacuoles that coalesced into lumens (cross-section). Angiogenesis (right panels); ECs were plated on top of the collagen matrices. The black arrows indicate the monolayer of ECs. The white arrows indicate EC tubes and sprouts (tip cells) from tube-like structures on the monolayer. Bar=25μm. Reprinted with

permission from “Mechanisms controlling human endothelial lumen formation and tube assembly in three-dimensional extracellular matrices” by Davis et al., 2007, Birth Defects Res C Embryo Today, 81:270-285 (Davis et al., 2007).

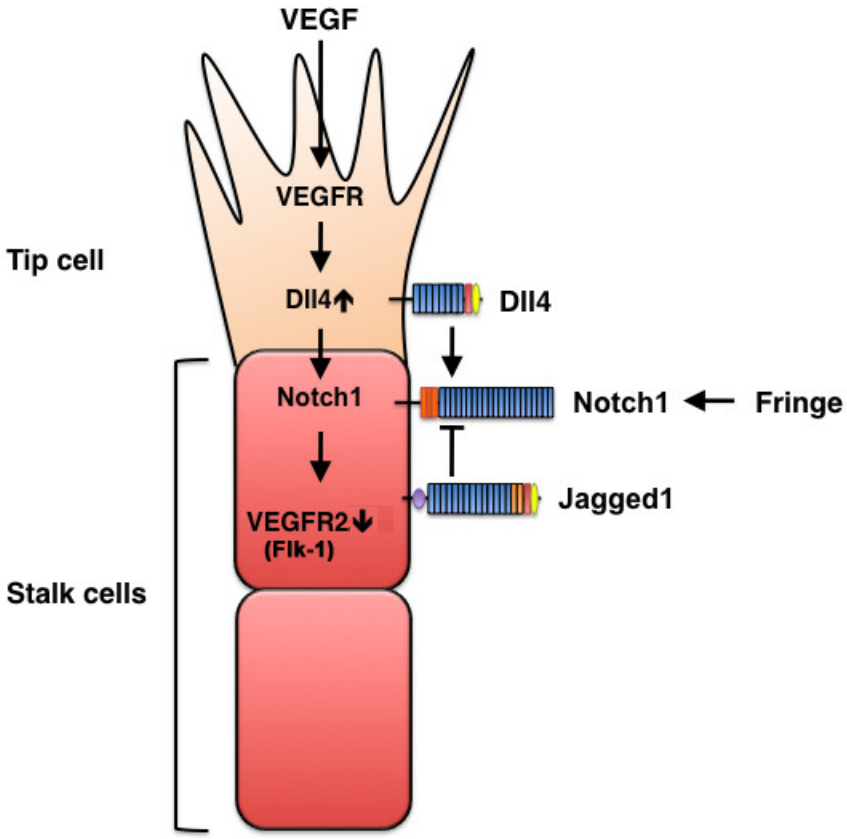


Figure 1.3. Endothelial tip and stalk cells in angiogenesis. VEGF from surrounding tissue binds to VEGF receptor on tip cells and stimulate expression of Dll4. Dll4 activates Notch signaling (particularly Notch1) in stalk cells to reduce stalk cell sensitivity to VEGF stimulation and suppress the tip-cell phenotype. In contrast, Jagged1 expressed in stalk cells prevents expression of Dll4 that activate Notch1 in neighboring ECs (stalk or tip cells) to increase tip cell numbers. Antagonistic interaction between Dll4 and Jagged1 in ECs is mediated by Fringe (glycosyltransferase), which enhances Notch activation in response to Dll4 and reduces Notch activity in response to Jagged1. Reprinted with permission from "Novel insights into the differential functions of Notch ligands in vascular formation" by Kume, 2009, J Angiogenes Res 1:8 (Kume, 2009).

Notch signaling in angiogenesis

Notch receptors and their ligands are essential for sprouting angiogenesis during mouse development (Hellstrom et al., 2007; Lobov et al., 2007; Noguera-Troise et al., 2006; Ridgway et al., 2006; Sainson et al., 2005; Suchting et al., 2007). Notch signaling is evolutionarily conserved and involved in various developmental processes. Notch receptor family members and Notch ligands are expressed throughout early vascular development (Gridley, 2007, 2010). The mammalian Notch signaling pathway is composed of four Notch receptors (Notch1-4) and five ligands (Jagged1 and 2 and Delta-like (Dll) 1, 3, and 4) (Gridley, 2007, 2010). All of the ligands are transmembrane and interact with Notch receptors on adjacent cell surfaces by cell-cell interactions. After ligand binding, the Notch receptor is cleaved by γ-secretase and the Notch intracellular domain (NICD) is released into the cytoplasm. The NICD translocates into the nucleus and forms a transcriptional activation complex that promotes downstream target gene transcription. Both Notch ligands and receptors are expressed in vascular endothelial cells and Notch signaling is known to be involved in vascular development (Alva & Iruela-Arispe, 2004; Gridley, 2010; Hofmann & Iruela-Arispe, 2007; Phng & Gerhardt, 2009; Roca & Adams, 2007). Notch1 is broadly expressed in many tissues including vascular ECs while Notch4 expression is restricted to vascular ECs (Krebs et al., 2000; J. Wu & Bresnick, 2007; J. Wu et al., 2005), and Notch3 is predominantly expressed in vascular smooth muscle cells (Joutel et al., 2000). Notch ligands (Jagged1, Jagged2, Dll1, and Dll4) are expressed in vascular ECs (Beckers, Clark, Wunsch,

Hrabe De Angelis, & Gossler, 1999; Krebs et al., 2000; Villa et al., 2001). Notch1$^{-/-}$, Notch1$^{-/-}$/Notch4$^{-/-}$, Jagged-1$^{-/-}$, and Dll1$^{-/-}$ mice display vascular defects (Hrabe de Angelis, McIntyre, & Gossler, 1997; Huppert et al., 2000; Kotani et al., 1999; Xue et al., 1999). Of interest, Notch1$^{-/-}$ and Notch1$^{-/-}$/Notch4$^{-/-}$ mouse embryos form a normal vascular plexus but fail to remodel the plexus to form conduit blood vessels at E9.5 (Huppert et al., 2000; Krebs et al., 2000). Similar to the Notch1$^{-/-}$ and Notch1$^{-/-}$/Notch4$^{-/-}$ mouse, deletion of the Dll4 gene reveals vascular remodeling defects (Duarte et al., 2004; Gale et al., 2004). Reduced level of Dll4 or blocking of Notch signaling promotes the formation of tip cells that lead to sprouting, branching, and fusion of endothelial tubes (Leslie et al., 2007; Siekmann & Lawson, 2007). When Dll4 and Notch signaling is compromised in zebrafish development, endothelial sprouts failed to display angiogenic motility and this caused excessive branching (Leslie et al., 2007; Siekmann & Lawson, 2007). Thus, Notch signaling is essential for angiogenic vascular morphogenesis and capillary plexus remodeling.

1.2. Endothelial Colony Forming Cells (ECFCs)

Recruitment of circulating endothelial progenitor cells (EPCs) to growing endothelial sprouts may combine vasculogenic and angiogenic mechanisms. The dorsal aorta and cardinal vein in the early embryo are formed by vasculogenesis, which is direct assembly of angioblasts and ECs. Similarly, in the adult organism, EPCs play critical roles in the formation of new vessels or the recovery of damaged vascular endothelium through angiogenesis and vasculogenesis

(Asahara et al., 1997; Rafii, Lyden, Benezra, Hattori, & Heissig, 2002; Shi et al., 1998). While the majority of circulating human ECs are senescent sloughed cells undergoing anoikis (Woywodt, Erdbruegger, & Haubitz, 2006), reports over 5 decades have reported evidence that some circulating mammalian ECs attach and proliferate to form colonies of endothelium that coat intravascular suspended biomaterials (Stump, Jordan, Debakey, & Halpert, 1963). In 1997, Asahara reported that circulating endothelial progenitors could be isolated from human adult peripheral blood and lead to revascularization *in vivo* in response to acute tissue ischemia (Asahara et al., 1997). EPCs have been subsequently reported to contribute to new vessel formation in adult subjects (Asahara et al., 1997; Shi et al., 1998). This process is termed neovasculogenesis or postnatal vasculogenesis: the formation of new vessels by circulating ECs in the adult (combination of angiogenesis and vasculogenesis).

We have isolated endothelial colony forming cells (ECFCs) from human peripheral blood and cord blood (Au, Daheron, et al., 2008; Ingram et al., 2004; Melero-Martin et al., 2007). Human mononuclear cells (MNCs) are placed on rat-tail collagen I coated dishes and the nonadherent cells are removed after one day. Between 5 and 14 days, colonies of ECs appear and are identified as typical cobblestone morphology in endothelial cell growth medium. ECFCs express cell surface proteins (KDR, CD34, vWF, eNOS, VE-cadherin, and others) similar to those on primary ECs, but ECFCs do not express hematopoietic cell markers such as CD45, AC133, CD11b, and CD14 (Ingram et al., 2004; Yoder et al.,

2007). ECFCs derived from human adult and cord blood are able to incorporate DiI-labeled acetylated-low density lipoprotein (acLDL) (Ingram et al., 2004). They are highly proliferative and form capillary-like structures *in vitro* (Ingram et al., 2004). They can proliferate at a clonal level and be replated into secondary and tertiary ECFCs (Ingram et al., 2004). When single ECFCs were plated in defined conditions, they displayed a complete hierarchy of clonal proliferative potential (Figure 1.4. a and b). The highest proliferative potential (HPP)-ECFCs, formed the largest colonies and displayed replating potential, low proliferative potential (LPP)-ECFCs formed smaller colonies without replating potential, endothelial clusters that gave rise only to non-proliferating mature cells, and non-proliferative ECs lack ECFC activity. Most importantly, in contrast to colony forming unit-Hill (CFU-Hill) and any other putative EPCs, ECFCs are able to form human blood vessel de novo *in vivo* when implanted in immunodeficient mice (Yoder et al., 2007) (Figure 1.4. C). The human ECFC-derived capillary plexus inosculates with the murine vessels to become the part of the host blood vascular system (Yoder et al., 2007) (Figure 1.4. D). Thus, only ECFCs display characteristics of high proliferative potential and *in vivo* vessel forming ability.

Blood vessel damage increases the number of circulating EPCs in patients with peripheral artery disease (PAD) (Hill et al., 2003; Rafii & Lyden, 2003; Urbich & Dimmeler, 2004). Some studies suggest that certain subsets of circulating EPCs may monitor the status of endothelium and be recruited into a site of vascular injury to facilitate angiogenesis (Urbich & Dimmeler, 2004; Werner et al., 2005).

Especially, ECFCs have shown promise for tissue regeneration. In mouse vascular injury models, ECFCs are recruited and incorporate into injured vessels or ischemic tissue (Schwarz et al., 2012). Several studies have reported that ECFCs enhance vascular repair, improve blood flow after ischemic retinopathy, limb ischemia, myocardial infarction, and stroke (X. T. Huang et al., 2013; Kang, Coggins, Xiao, Rosenzweig, & Bischoff, 2013; Medina, O'Neill, Humphreys, Gardiner, & Stitt, 2010; Moubarik et al., 2011; Schwarz et al., 2012; Stroncek, Ren, Klitzman, & Reichert, 2012). ECFCs also engraft and re-endothelialized denuded vascular segments or implanted grafts (Stroncek et al., 2012). Thus, introduction of ECFCs into patients could have major therapeutic benefits.

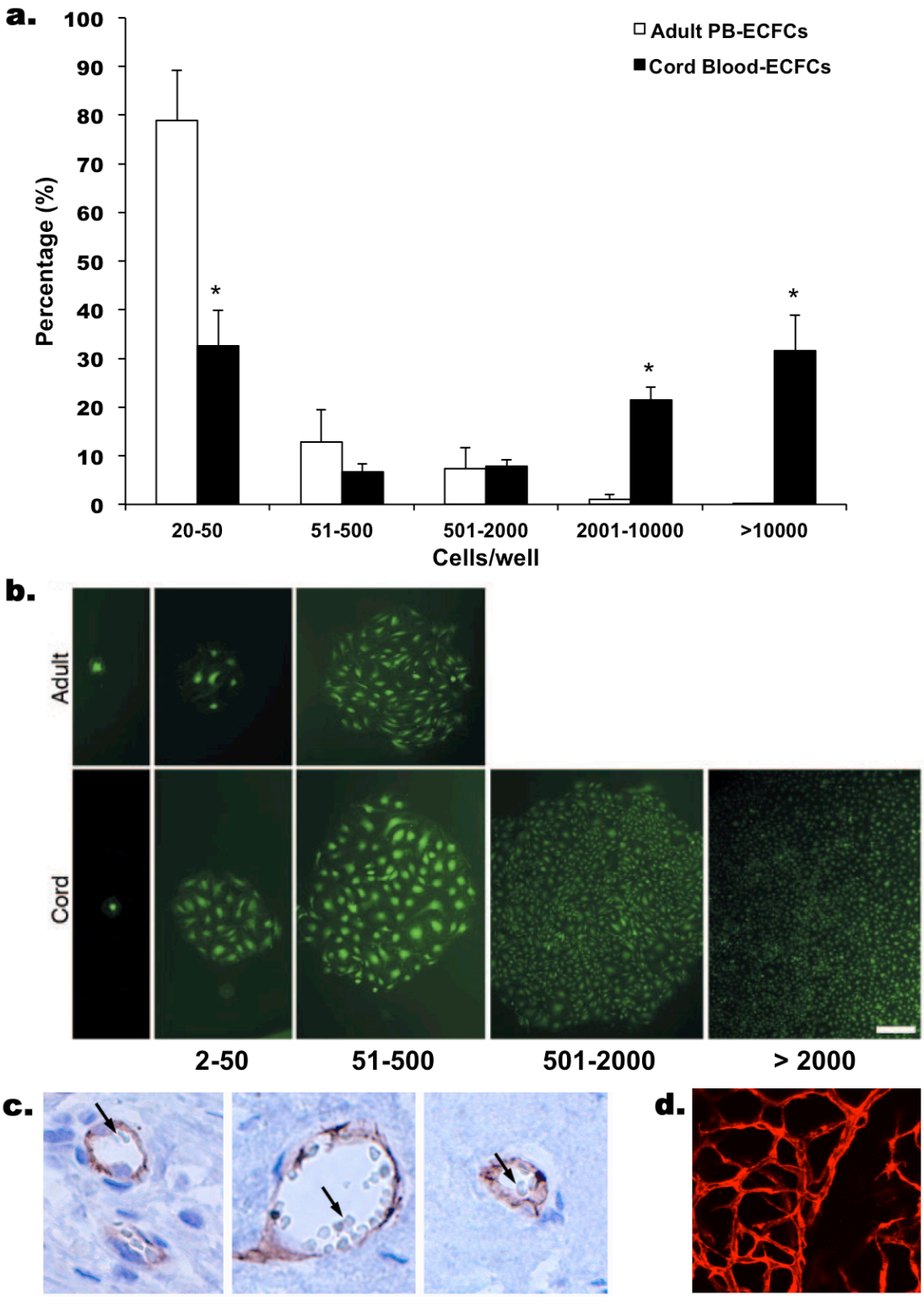

a.
Adult PB-ECFCs
Cord Blood-ECFCs
Percentage (%)
100
90
80
70
60
50
40
30
20
10
0
*
*
*
20-50
51-500
501-2000
2001-10000
>10000
Cells/well
b.
Adult
Cord
2-50
51-500
501-2000
> 2000
c.
d.

Figure 1.4. Quantitation of the clonogenic and proliferative potential of single ECs derived from cord blood and adult peripheral blood (PB) and vessel formation properties of ECFCs. (a) The percentage of single ECFCs dividing at least once after 14 days culture. (b) Representative photomicrographs (50x magnification) of the different size of endothelial cell clusters or colonies. (c) Formation of functional blood vessels *in vivo* (arrow indicates murine red blood cells in the lumen of human vessel) when implanted into immunodeficient mice. (d) Moreover, these vessels inosculate with the murine vessels and become the part of host vascular system. Reprinted with permission from "Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood" by Ingram et al., 2004, Blood, 104:2752-2760 and "Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals" by Yoder et al., 2007, Blood, 109:1801-1809 (Ingram et al., 2004; Yoder et al., 2007).

1.3. Akt Signaling

Protein kinase B (PKB), also known as Akt is a member of the serine/threonine family of protein kinases and is comprised of three isoforms, Akt1, Akt2, and Akt3. Akt is critical for neovasculogenesis since it is a downstream target of pro-angiogenic growth and survival factors including VEGF (Fujio & Walsh, 1999; Gerber et al., 1998). Activation of Akt leads to a number of changes in EC behavior including enhanced angiogenesis, vasculogenesis, growth, and regulation of apoptosis/cellular survival (Llevadot & Asahara, 2002).

Akt phosphorylates endothelial nitric oxide (NO) synthase (eNOS), enhances endothelial NO synthesis, and influences postnatal vessel growth (Dimmeler et al., 1999; Fulton et al., 1999). Akt signaling can mediate the endothelial response to angiogenic stimuli, including NO production and VEGF-induced migration in ECs and EPCs (Morales-Ruiz et al., 2000). Endothelial-specific Akt1 loss causes significant retinal vascular defects (M. Y. Lee et al., 2014). Global Akt1 deletion in murine pups increases neonatal mortality but some pups are viable despite growth retardation because of compensation from other Akt isoforms. Interestingly, both $Akt1^{-/-}/Akt2^{-/-}$ double knockout (DKO) and $Akt1^{-/-}/Akt3^{-/-}$ DKO mice die during the embryonic or postnatal period (Peng et al., 2003; Z. Z. Yang et al., 2005). Furthermore, triple knockout mice ($Akt^{+/-}/Akt2^{-/-}/Akt3^{-/-}$) are viable with impaired growth (Dummler et al., 2006). These studies suggest a single Akt1 allele is sufficient for embryonic development and postnatal survival.

Akt has been known to promote the expression of anti-apoptotic B-cell lymphoma 2 (Bcl-2) proteins, such as B-cell lymphoma extra large (Bcl-xL) through the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (I. Kim et al., 2000; Zong, Edelstein, Chen, Bash, & Gelinas, 1999). Constitutive activation of Akt signaling protects cardiomyocytes against apoptosis (Fujio, Nguyen, Wencker, Kitsis, & Walsh, 2000). In addition, chronic activation of Akt pathway in ECs promotes cell survival of endothelial cells (ECs) and stimulates vasculogenic activity of ECs in absence of growth factors and serum (Seandel et al., 2008).

1.4. Human Platelet Lysate (HPL)

Human platelet lysate (HPL) contains various cytokines, chemokines, and growth factors that are involved in various cellular events, particularly cellular migration, proliferation, differentiation, extracellular matrix organization and remodeling, cell survival, and angiogenesis/neovascularization (tissue repair) (Aarabi, Longaker, & Gurtner, 2007; Crowley, Dempsey, Horwitz, & Horwitz, 1994; Fekete et al., 2012; Gurtner, Werner, Barrandon, & Longaker, 2008; Langer et al., 2009; Shih et al., 2011; Singer & Clark, 1999). Chemokines in HPL including stromal-derived growth factor 1 (SDF-1), stimulate recruitment, adhesion, and proliferation of $CD34^+$ progenitor cells, mesenchymal stromal cells (MSC), smooth muscle progenitors, and endothelial progenitors (de Boer et al., 2006; Langer et al., 2009; Lev et al., 2006; Zernecke et al., 2005). Growth factors in HPL, especially, VEGF, FGF, and angiopoietin-1, stimulate Akt to enhance cell survival and

ensure adequate vascular development in cardiovascular functions (Carmeliet et al., 1999; Fujio & Walsh, 1999; Gerber et al., 1998; I. Kim et al., 2000; Kontos et al., 1998; Papapetropoulos et al., 2000). In addition, other molecules such as SDF-1, hepatocyte growth factor (HGF), and sphingosine-1-phosphate promote survival signals for vascular ECs and smooth muscle cells (SMCs) at injured vascular sites (Hisano et al., 1999; Nakamura, Teramoto, & Ichihara, 1986; Stellos & Gawaz, 2007). HPL also contains platelet microparticles (PMPs) that are membrane vesicles carrying platelet-derived products, including chemokines, cytokines, and various bioactive lipids and are elevated in patients with atherosclerosis, acute vascular syndromes, and diabetes mellitus (Barry, Pratico, Savani, & FitzGerald, 1998; Mause, von Hundelshausen, Zernecke, Koenen, & Weber, 2005; Rozmyslowicz et al., 2003; VanWijk, VanBavel, Sturk, & Nieuwland, 2003). PMPs have been reported to activate Akt via phosphorylation and improve endothelial regeneration in murine vascular injured model (Mause et al., 2010). Moreover, PMPs phosphorylate and activate Akt that inactivate pro-apoptotic molecule B-cell lymphoma 2-associated death promoter (BAD) and stimulate anti-apoptotic pathways in a human monocytic leukemia cell line (Datta et al., 1997; Vasina et al., 2013). However, incubation of ECs and SMCs with PMPs from septic patients results in stimulation of apoptosis in the cells via production of reactive oxygen species (Gambim et al., 2007; Janiszewski et al., 2004). Therefore, HPL obtained from normal healthy subjects is involved in regulation of apoptosis and cell survival in numerous cells involved in the repair

of injured tissues, whereas HPL from septic patients may harbor deleterious signals to cell survival.

1.5. Apoptosis

Apoptosis is a highly conserved and essential cellular process in tissue development and homeostasis (Prindull, 1995). Characteristic apoptotic features are cell membrane blebbing, cell shrinkage, chromatin condensation, and DNA fragmentation, finally ending with the engraftment by tissue macrophages or other neighboring cells (Savill & Fadok, 2000). Apoptosis generally culminates with the sequential activation of caspases, the cysteine proteases for cleavage of proteins (Duprez, Wirawan, Vanden Berghe, & Vandenabeele, 2009; Sprick & Walczak, 2004; Taylor, Cullen, & Martin, 2008). Cells undergo apoptosis through two major pathways: the extrinsic pathway (death receptor pathway) is initiated by ligand binding to membrane receptors of the death receptor family, whereas the intrinsic pathway (mitochondrial pathway) is mediated by intracellular and extracellular stresses (Duprez et al., 2009; Sprick & Walczak, 2004; Taylor et al., 2008; Wyllie, 2010).

The extrinsic pathway involves the transmembrane death receptors of the tumor necrosis factor (TNF) receptor superfamily including FasR, TNFR1, and death receptors (DRs) (Duprez et al., 2009; Sprick & Walczak, 2004; Taylor et al., 2008; Wyllie, 2010). Death ligand stimulation leads to oligomerization of the receptors and recruitment of the adaptor protein, Fas-associated death domain

(FADD) and initiator caspases such as caspase-8 and -10 to form a death-inducing signaling complex (DISC). This leads to the activation of effector caspases, typically caspase-3. The intrinsic pathway is mediated by various physiochemical stresses such as chemotherapeutic agents, alterations in temperature, osmolality, DNA damaging agents, free radical generation compounds, removal of nutrients, and oxygen deprivation (Duprez et al., 2009; Sprick & Walczak, 2004; Taylor et al., 2008; Wyllie, 2010). The pathways lead to the same type of apoptotic response that causes release of cytochrome c (Cyt-C) from mitochondria into the cytoplasm and activation of caspase-3 (Duprez et al., 2009; Sprick & Walczak, 2004; Taylor et al., 2008). Caspase-3 is activated to cleaved caspase-3 to generate all the biochemical and morphologic hallmarks of cell apoptosis. Thus, the activation of the two pathways converges by activating common effector caspase cascades.

In normal cells, Akt activation (phospho-Akt, pAkt) by various growth factors stimulates cell survival via protection of mitochondrial integrity and inhibition of Cyt-C release (Gottlob et al., 2001; Plas, Talapatra, Edinger, Rathmell, & Thompson, 2001; Uchiyama, Engelman, Maulik, & Das, 2004; Wyllie, 2010). Akt has also been reported to promote the expression of the anti-apoptotic Bcl-2 proteins, such as Bcl-xL that inhibits activation of Bcl-2-associated X protein (Bax/Bak) to prevent permeablization of the mitochondrial membrane (Jones et al., 2000; Zong et al., 1999). However, in apoptotic cells, pro-apoptotic members (Bax, Bak, and Bad) are activated and induce the release of Cyt-C from

mitochondria by binding and inactivating anti-apoptotic proteins (Gustafsson & Gottlieb, 2007; Scorrano & Korsmeyer, 2003).

1.6. Summary

Formation of the vascular system, composed of functional arteries, veins, and capillaries, originates via two fundamental processes: vasculogenesis and angiogenesis (Adams & Alitalo, 2007). Angiogenesis is a well-studied process, controlled by a number of well-known angiogenic mediators and pathways in both developing embryo and postnatal life (Adams & Alitalo, 2007). However, identifying the molecular mechanisms that regulate and facilitate vasculogenesis has been less well examined.

We and others have identified rare viable circulating ECFC (Au, Daheron, et al., 2008; Ingram et al., 2004; Melero-Martin et al., 2007). Human cord blood (CB) derived circulating ECFC display a hierarchy of clonogenic proliferative potential and posses *de novo* vessel forming ability upon implantation in immunodeficient mice. Human ECFC-derived vessels form after implantation in NOD/SCID mice, but inosculation and perfusion by the host circulatory system does not occur until 3-4 days after implantation (P. Allen, K. T. Kang, & J. Bischoff, 2013). We have previously reported that only 1-3% of the total implanted human CB ECFC were alive following implantation *in vitro* and *in vivo* (H. Kim, Huang, et al., 2015). Thus, one fundamental question we wish to examine is how to improve cell survival of ECFC and promote vasculogenesis of ECFC in *in vitro* and *in vivo* models. Since

systemic blood flow is required to stabilize newly formed vessels, the overall focus of this dissertation is on the methods and mechanisms to enhance vasculogenesis of ECFC implanted *in vivo* to form a stable perfused capillary network that can be perfused by the host circulatory system.

CHAPTER 2. NOTCH LIGAND DELTA-LIKE 1 (DLL1) PROMOTES *IN VIVO* VASCULOGENESIS IN HUMAN CORD BLOOD DERIVED ENDOTHELIAL COLONY FORMING CELLS (ECFCS)

The following chapter is reprinted with permission from "Notch ligand Delta-like 1 promotes *in vivo* vasculogenesis in human cord blood-derived endothelial colony forming cells" by Kim et al., 2015, Cytotherapy, 17(5):579-92 (H. Kim, Huang, et al., 2015).

2.1. Introduction

The Notch signaling pathway is an evolutionarily conserved pathway that is involved in a variety of developmental processes (Ables, Breunig, Eisch, & Rakic, 2011; Niessen & Karsan, 2008; Radtke, Fasnacht, & Macdonald, 2010). Notch family members and Notch ligands are expressed in endothelial cells (ECs) throughout early vascular development (Kume, 2009; Rocha & Adams, 2009; Swift & Weinstein, 2009). Notch1, Notch4, Jagged2, Dll1, and Dll4 are specifically expressed in arterial endothelium (Kume, 2009; Takeshita et al., 2007) and these molecules play critical roles in arterial specification (Adams & Alitalo, 2007; Alva & Iruela-Arispe, 2004; Rossant & Howard, 2002; Shawber & Kitajewski, 2004; Siekmann, Covassin, & Lawson, 2008; Sorensen, Adams, & Gossler, 2009). VEGF is one of the most potent and ubiquitous vascular growth factors that affect many aspects of EC biology. Recently, VEGF-A has been found to interact with the activated Notch pathway to determine and maintain

arterial EC fate (Lawson, Vogel, & Weinstein, 2002; Sorensen et al., 2009; Zhong, Childs, Leu, & Fishman, 2001). Less is known of how Notch signaling may influence the vasculogenic properties of human vessel forming cells.

In Notch1 and 4 deficient mouse embryos, embryos failed to remodel the plexus to form large and small blood vessels, although the primary vascular plexus forms normally. These data indicate that Notch signaling plays a critical role in angiogenic vascular morphogenesis and remodeling (Huppert et al., 2000; Krebs et al., 2000). Different human endothelial cell lines have been used to study, the regulation of Notch signaling in human vascular development. Most studies have utilized human umbilical vein endothelial cells (HUVECs) or human arterial/microvascular endothelial cells to investigate the roles of the Notch signaling pathway on their angiogenic and vasculogenic behavior. Notch1 activation has been implicated both in promoting and in inhibiting cell death in a cell type specific manner (Beverly, Felsher, & Capobianco, 2005; Jehn, Bielke, Pear, & Osborne, 1999; MacKenzie, Duriez, Wong, Noseda, & Karsan, 2004; Sade, Krishna, & Sarin, 2004). Notch1 activation in cultured monocytes plated upon immobilized $Delta^{ext\text{-}myc}$ has been reported to induce apoptosis (Ohishi et al., 2000). On the other hand, activation of Notch 1 and a downstream mediator, HES1, in human iliac artery endothelial cells (HIAECs) caused growth suppression but improved cell survival of the cultured cells. Also, activated Notch1 in HIAECs formed more stabilized networks and cord formation on Matrigel substrate in the presence of VEGF (Z. J. Liu et al., 2003). Dll1-

dependent Notch signaling mediated by EphrinB2 induced branching morphogenesis and network formation by human arterial endothelial cells (HAECs) plated on Matrigel (Limbourg et al., 2007). These reports indicated that Notch1 signaling plays a role in regulating endothelial cell survival, network, and cord formation *in vitro*, but limited evidence has been presented to examine how *in vitro* Notch activation preconditioning might influence angiogenic or vasculogenic behavior when cells are implanted *in vivo*.

We have successfully isolated circulating endothelial colony forming cells (ECFCs) from human umbilical cord blood (CB) and identified a hierarchy of proliferative potential in ECFC through the use of single cell clonogenic and functional assays (L. Huang, Critser, Grimes, & Yoder, 2011; Ingram et al., 2004). Human cord blood ECFCs form a human capillary plexus in immunodeficient mice after subcutaneous implantation in collagen-fibronectin matrices and upon inosculation with murine vessels, become a part of the systemic host circulation (L. Huang et al., 2011; Yoder et al., 2007). We recently determined that all viable circulating ECFC in human cord blood (CB) and adult peripheral blood can be enriched and identified as endothelial cells expressing CD34, CD146, CD105, CD31, but not CD45 or CD133 (Mund, Estes, Yoder, Ingram, & Case, 2012). These circulating viable endothelial cells represent those rare circulating ECFC that are known to colonize and re-endothelialize implanted biomaterials in human subjects (Hirschi, Ingram, & Yoder, 2008; Stump et al., 1963).

The use of circulating CB ECFC to study human vessel formation within immunodeficient mice permits analysis of human vasculogenesis and determination of key regulatory molecules controlling human vessel formation *in vivo* in a unique model system. For example, use of cultured CB ECFC with adult bone marrow mesenchymal stromal cells (MSC) can recreate a hematopoietic and leukemic stem cell niche *in vivo* (Y. Chen et al., 2012). Since Notch pathway activation plays such a key role in establishing the murine embryonic vascular system, we hypothesized that *in vitro* preconditioning of human CB ECFC with Notch ligand may enhance *in vivo* vasculogenic activity. We demonstrate that preconditioning of ECFC with Notch activation *in vitro* is insufficient to promote *in vivo* vasculogenesis, however, provision of the Notch ligand Dll1 by OP9 stromal cells *in vivo* activates Notch1 signaling in ECFC and enhances human blood vessel formation.

2.2. Materials And Methods

Media and supplements

Human endothelial serum free medium (SFM, Invitrogen) was supplemented with 20 ng/ml human recombinant basic fibroblast growth factor (hrbFGF, Invitrogen), 10 ng/ml human recombinant epidermal growth factor (hrEGF, R&D), 10 ng/ml human recombinant vascular endothelial growth factor 165 (rhVEGF-A/rhVEGF$_{165}$, R&D), 10 ng/ml rhVEGF$_{121}$ (R&D), 10 ng/ml stem cell factor (SCF, R&D), 5 ng/ml stromal cell derived factor 1alpha (SDF1α, R&D), 10 ng/ml

interleukin 6 (IL6, R&D) and 1.5% human cord plasma (HCP), to create serum reduced medium (SRM).

Isolation and culture of human umbilical cord blood (CB) derived ECFCs

Human CB samples (50–100 mL) were collected in heparin-coated syringes from healthy newborns (38–40 weeks gestation). The Institutional Review Board at Indiana University School of Medicine reviewed and approved this study with exempt IRB status. UCB was diluted 1:1 with Dulbecco's phosphate buffered saline (DPBS, Invitrogen) and overlaid onto Ficoll-Paque PLUS (GE Healthcare). Cells were centrifuged for 30 minutes at room temperature (RT) at 1500 rpm. Mononuclear cells (MNCs) were isolated and washed with DPBS. For outgrowth of ECFC colonies, MNCs were resuspended in SRM. 3×10^7 MNCs were seeded onto each well of 6-well tissue culture plates pre-coated with Type I rat-tail collagen (BD Biosciences Pharmingen) and cultured as previously described (Ingram et al., 2004). ECFC colonies appeared around 4 days of culture and were noted to form colonies of adherent cells with cobblestone morphology. After approximately 10 days of culture, the ECFC-derived ECs were released from the culture dish by TrypLE™ Express (Gibco) and replated onto 25 cm^2 tissue culture flasks pre-coated with Type I rat-tail collagen for subsequent passage. Characterization of human UCB ECFC derived ECs was conducted using monoclonal antibodies and fluorescence-activated cell sorter (FACS) analysis as previously described (Ingram et al., 2004).

Immobilization of Delta1$^{ext\text{-}IgG}$ protein

Delta1$^{ext\text{-}IgG}$ protein is the extracellular domain of human delta-like 1(Dll1) fused to the Fc domain of human IgG1 (Varnum-Finney et al., 2000). Non-tissue culture-treated plates were coated with decreasing concentrations of Delta1$^{ext\text{-}IgG}$ (20, 10, 5, 2.5, 1.25, 0.625, and 0.3125 μg/mL) or the same concentration of human IgG (Sigma-Aldrich), diluted in PBS together with 5 μg/mL fibronectin fragment CH-296 (Takara Shuzo). The plates were incubated overnight at 4°C, washed with PBS 3 times, and further incubated with 2% bovine serum albumin (BSA) dissolved in PBS at 37°C for 1 hour. Thereafter, plates were washed with PBS 3 times and were then ready for plating cells.

RNA isolation and conventional/quantitative RT-PCR

Total cellular RNA was extracted with an RNeasy Micro extraction kit (Qiagen) as described by the manufacturer. RT reactions were performed using an Omniscript RT Kit (Qiagen). Conventional PCR was conducted by using Go Tap Flexi DNA Polymerase (Promega) according to the manufacturer's instructions. The primer sequences are shown in Table 2.1. The PCR cycle profile was 94°C for 5 minutes, 94°C for 30 seconds, 53 or 57°C (depending on the different primers) for 30 seconds, 72°C for 45 seconds, and 32 cycles with a final 72°C for 7 minute. PCR products were added to wells in a 2% agarose/ethidium bromide gel and exposed to electrophoresis current. Migrating bands were photographed under UV light. Quantitative PCR was performed using FastStart Universal SYBR Green Master 2x (Rox) (Roche). The relative standard curve of each gene

amplification was first generated to determine the amplification efficiency (Eff). ATP5B was used as a housekeeping gene. To compare gene expression levels among treated and control ECFCs, results were presented as the ratio of the expression of each gene to ATP5B expression. For Delta1$^{ext\text{-}IgG}$ or γ-secretase inhibitor L685458 treatment effects on ECFCs, gene expression levels in non-treated cells at Day 0 were analyzed as controls. Results were expressed as a fold change (in logarithmic scale) in comparison to the control. The quantitative analysis was performed according to Pfaffl's method (Pfaffl, 2001). The primer sequences are shown in Table 2.2.

Table 2.1. Primers used for conventional RT-PCR

Gene	Forward	Reverse	Tm	size (bp)
VEGF	AGTTTAAAAGGCACCCAGCA	ACGAGCTCCCTTCCTTCAGT	55	362
VEGFR2	GAGGGACTTGGACTGGCTTT	GATTTGAAATGGACCCGAGA	55	302
VEGFR3	TGAACATCACGGAGGAGTCA	TCAGGCTTGTTGATGAATGG	55	337
NRP1	GAAAAATGCGAATGGCTGAT	AATCCGGGGGACTTTATCAC	53	335
NRP2	CAAACACTGTGGGAACATCG	TGTCCAGCCAATCGTACTTG	55	338
DLL1	TGTGCCTCAAGCACTACCAG	ACACACGAAGCGGTAGGAGT	55	353
DLL4	TATTGGGCACCAACTCCTTC	ACATAGTGGCCGAAGTGGTC	55	349
NOTCH1	GGCCAGAACTGTGAGGAAAA	GCAGTAGAAGGAGGCCACAC	57	327
NOTCH4	TCTCCCTCTCCATTGACACC	TGGAAGCACTCGTTGACATC	55	323
HEY2	TGGGGAGCGAGAACAATTAC	GCACTCTCGGAATCCTATGC	55	329
EFNB2 (C)	TTATTTGCCCCAAAGTGGAC	CCTGGTTGATCCAGCAGAAC	55	347
EPHB4	GAGCTGTGTGGCAATCAAGA	ACTTTGCAGACGAGGTTGCT	55	345
COUPTFII	AACACATCGAGTGCGTGGT	CAGGTACGAGTGGCAGTTGA	55	311
PECAM1	GCAAAATGGGAAGAACCTGA	ACAGTTGACCCTCACGATCC	55	316
VECAD	TGGACAAGGACACTGGTGAA	TCTTGCAGAGTGACCAGCAC	55	382
ACTB	GCCAGCTCACCATGGATGAT	GTCTCAAACATGATCTGGGTC	57	388

Reprinted with permission from “Notch ligand Delta-like 1 promotes *in vivo* vasculogenesis in human cord blood-derived endothelial colony forming cells” by Kim et al., 2015, Cytotherapy, 17(5):579-92. Table 2.1. was drawn by my co-worker, Lan Huang (H. Kim, Huang, et al., 2015).

Table 2.2. Primers used for quantitative RT-PCR

Gene	Forwards	Reverse	Eff
VEGFR1	GCTTCTGACCTGTGAAGCAA	CTCGTGTTCAAGGGAGTGGT	1.987
VEGFR2	GAACATTTGGGAAATCTCTTGC	CGGAAGAACAATGTAGTCTTTG	1.917
VEGFR3	AGACAAGAAAGCGGCTTCAG	TTGGGAGTCAGGGTGTGC	1.860
NRP1	GTTGTGTCTTCAGGGCCATT	AATCCGGGGGACTTTATCAC	2.036
NRP2	TCTGCGCTACGAGATCTTCA	GTGCAGTCCAAGTTGTGTGG	1.862
DLL4	GACCACTTCGGCCACTATGT	TTGCTGCAGTAGCCATTCTG	1.967
NOTCH1	CTTCAATGACCCCTGGAAGA	GAAGTGGAAGGAGCTGTTGC	1.950
NOTCH4	CTGCTGCTGCTGCTATGTGT	GTCAGGAAACTGGCACGTCT	1.833
HES1	TGCTTCACTGTCATTTCCAGA	GAAAGTCTGAGCCAGCTGAA	1.958
HEY2	CTTGTGCCAACTGCTTTTGA	TCATGAAGTCCATGGCAAGA	1.912
EFNB2	TCTTTGGAGGGCCTGGAT	CCAGCAGAACTTGCATCTTG	1.986
EPHB4	TTTGGCTCCTTCGAGCTG	GGCCAAGATTTTCTTCTGGTG	1.880
COUPTF	CCAAGAGCAAGTGGAGAAGC	TCCACATGGGCTACATCAGA	1.988
ATP5B	CCACTACCAAGAAGGGATCTATCA	GGGCAGGGTCAGTCAAGTC	1.960

Reprinted with permission from “Notch ligand Delta-like 1 promotes *in vivo* vasculogenesis in human cord blood-derived endothelial colony forming cells” by Kim et al., 2015, Cytotherapy, 17(5):579-92. Table 2.2. was drawn by my co-worker, Lan Huang (H. Kim, Huang, et al., 2015).

Notch ligand and receptor cell surface expression on ECFC

Relative levels of Notch ligands and receptors on ECFCs were determined by flow cytometry. ECFCs were harvested by cell dissociation buffer enzyme-free Hanks'-based reagent (Gibco). Cells (0.5×10^6) were suspended in staining buffer (PBS, 0.5% bovine serum albumin BSA, and 2mM EDTA) and stained using purified anti-human Notch4 (clone MHN4-2, Isotype mouse IgG1κ, BioLegend), Biotin anti-human Notch1 (clone mN1A, BioLegend), anti-human Delta-like protein 1 (Dll1) conjugated to phycoerythrin (PE) (clone MHD 1-314, BioLegend), anti-human Delta-like protein 4 (Dll4) conjugated to APC (clone MHD 4-46, BioLegend), and anti-human CD31 antibody conjugated to phycoerythrin-cyanine7 (PE-Cy7) (clone WM-59, eBiosciences) for 15 minutes at room temperature in the dark. After staining with purified and biotin conjugated primary antibodies, cells were stained with secondary antibody, goat anti-mouse alexa fluor 488 (Invitrogen), streptavidin-allophycocyanin (APC) (eBioscience), and with propidium iodide (PI) (eBiosciences) for 15 minutes at room temperature in the dark. For negative controls, we used fluorescence minus one (FMO) controls, as we have previously described (Mund et al., 2012). Stained cells were analyzed by FlowJo software.

Western blot

Protein extracts were prepared as described previously (Z. Yang et al., 2009), electrophoresed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and probed with

purified anti-human CoupTFII (clone H7147, R&D), anti-human Hey2 (ab167280, Abcam), and anti-human β-actin (mAbcam 8226, Abcam). For Notch receptors, proteins were transferred to immobilon-P membrane, PVDF, 0.45 μm, (Millipore) and probed with purified anti-human Notch1 (clone C-20, Santa Cruz Biotechnology), anti-human Notch2 (clone 25-255, Santa Cruz Biotechnology), and anti-human Notch4 (clone H-225, Santa Cruz Biotechnology).

Implantation of human cord blood derived ECFCs into NOD/SCID mice

Cellularized matrix implants were cast as previously described (L. Huang et al., 2011; Yoder et al., 2007). Cultured ECFCs (2 x 10^6 cells/mL) were suspended in a solution containing 1.5 mg/mL rat-tail collagen I (BD Biosciences), 100 μg/mL human fibronectin (Chemicon), 1.5 mg/mL sodium bicarbonate (Sigma), 25 mM HEPES (Cambrex), 10% FBS, and 30% SFM, pH-adjusted to 7.4. Then 250 μL of the cell suspension was pipetted into one well of a 48-well tissue culture plate, allowed to polymerize at 37°C for 30 minutes, and covered with 500 μL SRM for overnight incubation at 37°C in 5% CO_2. Matrices were implanted into the flanks of anesthetized 6-9 week old NOD/SCID mice. After 14 days, the mice were sacrificed; the grafts were excised and analyzed by histology and immunohistochemistry (N=6) as previously described (L. Huang et al., 2011; Yoder et al., 2007). ECFCs were also co-implanted with OP9 or OP9-DL1 stromal cells in some studies. OP9-DL1 cells represent murine bone marrow stromal OP9 cells that have been engineered to constitutively express the Notch

ligand Dll1 (Schmitt & Zuniga-Pflucker, 2002). ECFCs and OP9 or OP9-DL1 were implanted at 4:1 ratios as above.

Histology and immunohistochemistry

Sections were stained as previously described (Yoder et al., 2007). Briefly, paraffin-embedded tissue sections were deparaffinized and then either directly stained with hematoxylin and eosin (H&E) or immersed in retrieval solution (Dako) for 20 minutes at 90–99°C. Slides were incubated at room temperature for 30 minutes with anti-human CD31 antibody (clone JC70/A, Abcam) and rat anti-mouse smooth muscle alpha actin (αSMA) (clone 1A4, Sigma) followed by a 10-minute incubation with LASB2 link-biotin and streptavidin-HRP (Vector Laboratories), then developed with DAB (Vector Laboratories) solution for 5 minutes. Slides were analyzed by microscope under 40x magnification using a Leica DM 4000B microscope (Leica Microsystems) with attached Spot-KE digital camera (Diagnostic Instruments).

Assessment of apoptosis of ECFCs in 3D collagen matrices

Apoptosis was assessed by examining the percentage of human CD31 positive ECFCs that bound Annexin V and propidium iodide and was performed as per the manufacturer's instruction (Apoptosis Detection kit, eBioscience). OP9 or OP9-DL1 cells were co-implanted with ECFCs (1:1) in collagen matrices at indicated time points as above. Collagen matrices were prepared as described previously (Critser, Kreger, Voytik-Harbin, & Yoder; Yoder et al., 2007). Briefly, 2

x 10^6 cells/mL were implanted in collagen solution with endothelial cell growth medium (EGM-2, Lonza). Matrices were recovered from one to three days later and incubated in 250 µl Collagenase Type I (0.25%) (Stemcell technology) for 20 minutes at 37°C. Cell dissociation buffer was added to stop the enzymatic reaction (Invitrogen). Cells were centrifuged at 500 g for 5 minutes at room temperature. Cell pellets were suspended in staining buffer and stained with anti-human CD31 antibody conjugated to PE for 15 minutes (clone WM-59, BD Biosciences Pharmingen). Cells were incubated in binding buffer for 20 minutes and stained for Annexin V-APC and propidium iodide (PI) for 30 minutes at room temperature in the dark. Stained cells were analyzed by FlowJo software.

Caspase-1 and -3/7 assays in 3D collagen matrices

Relative levels of active caspase-1 and -3/7 activities were determined by flow cytometry using fluorescein-labeled inhibitors of caspases (FLICA) reagent according to the manufacturer's instructions (FLICA 660 Caspase-3/7 assay kit and FLICA 660 *in vitro* Caspase-1 Detection kit, Immunochemistry Technology). Collagen matrices and cells were prepared as previously described in assessment of apoptosis above. Cells recovered from matrices were suspended in staining buffer and stained with anti-human CD31 antibody conjugated to PE (clone WM-59, BD Biosciences Pharmingen) for 15 minutes. Cells were incubated with the FLICA 660-DEVD-FMK caspse-3/7 inhibitor or FLICA 660-YVAD-FMK caspase-1 inhibitor reagent for 20 minutes at room temperature in the dark. Unbound reagents were removed by two washes in wash buffer. The

cells were stained with PI to assess viability. The cells were analyzed by LSR II flow cytometer (BD Biosciences Pharmingen) using FlowJo software.

Statistical analysis

Results are expressed as mean ± the standard error of the mean (SEM) for the study variables. The change of gene expression after rhVEGF-A, Delta1$^{ext\text{-}IgG}$, or γ-secretase inhibitor L685458 induction in ECFC was assessed by Student's paired t-test. The vessel number and size distribution were evaluated by Students unpaired t-test. A statistically significant difference was set at $P<0.05$. The quantification of western blot data points, the number of αSMA^{+} vessels, the assessment of apoptosis of ECFCs, and results of caspase-1 and -3/7 assays in 3D collagen matrices were assessed by One-way ANOVA, *$P<0.05$, **$P<0.001$, or ***$P<0.0001$.

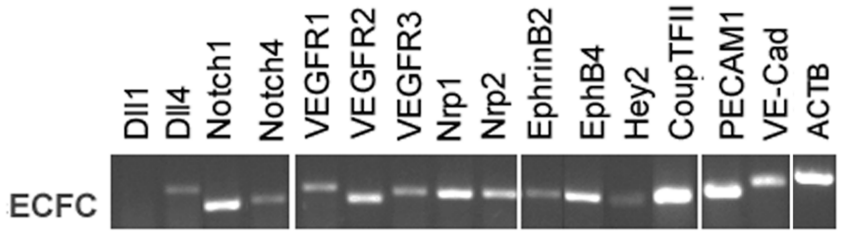
2.3. Results

Cultured human cord blood ECFC expresses multiple Notch pathway transcripts

We examined cultured human cord blood ECFCs to determine if Notch ligand/receptor or downstream genes activated by Notch ligand binding were evident at baseline conditions, along with some typical endothelial transcripts. Cultured ECFCs expressed a variety of the Notch pathway related transcripts (Figure 2.1. a). ECFCs displayed several typical endothelial cell surface markers such as platelet endothelial cell adhesion molecule-I (PECAM-I, CD31) and

vascular endothelial-cadherin (VE-Cad), which are typical of an EC identity and these cells have all been shown to give rise to cultures of ECs that display *in vivo* human vessel forming potential (Ingram et al., 2005; Yoder et al., 2007). We also demonstrated protein expression of Notch ligands and Notch receptors on ECFCs by flow cytometry (Figure 2.1. b). While Dll1 was not detected on ECFCs, Notch1 (2.7 ± 0.5%), Notch4 (2.9 ± 0.3%), and Dll4 (4.6 ± 0.9%) were expressed on subsets of ECFCs. N=3.

a.


Dll1
Dll4
Notch1
Notch4
VEGFR1
VEGFR2
VEGFR3
Nrp1
Nrp2
EphrinB2
EphB4
Hey2
CoupTFII
PECAM1
VE-Cad
ACTB
ECFC

b.

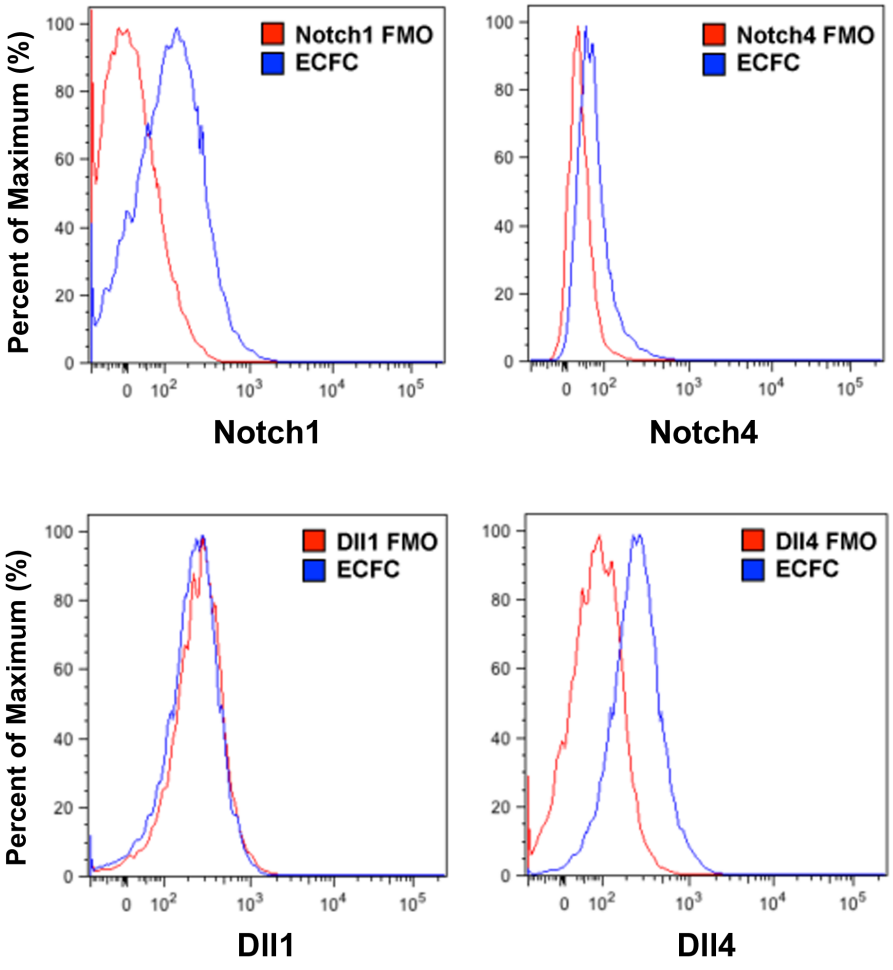

Notch1 FMO
ECFC
Percent of Maximum (%)
Notch1
Notch4 FMO
ECFC
Notch4
Dll1 FMO
ECFC
Percent of Maximum (%)
Dll1
Dll4 FMO
ECFC
Dll4

Figure 2.1. The expression of Notch pathway related transcripts in cultured ECFCs. (a) Human cord blood (CB) derived ECFCs expressed numerous Notch pathway related transcripts and EC gene markers such as PECAM-I and VE-Cadherin. (b) Notch ligands and receptors were expressed on a subset of ECFCs. The representative histograms show comparison between fluorescence minus one (FMO) control (red) and Notch molecules (blue) on ECFCs. Reprinted with permission from "Notch ligand Delta-like 1 promotes *in vivo* vasculogenesis in human cord blood-derived endothelial colony forming cells" by Kim et al., 2015, Cytotherapy, 17(5):579-92. Figure 2.1. (a) was performed by my co-worker, Lan Huang. Figure 2.1. (b) was from my original work (H. Kim, Huang, et al., 2015).

Delta1$^{ext\text{-}IgG}$ induces known Notch downstream target gene transcripts in human cord blood ECFCs in a dose-dependent manner

Previous studies have indicated that Notch signaling is required for blood vessel formation during embryo development (Duarte et al., 2004; Gale et al., 2004; Lawson et al., 2001; Rocha & Adams, 2009; Swift & Weinstein, 2009). In this study, the engineered Notch ligand, Delta1$^{ext\text{-}IgG}$, consisting of the extracellular domain of Dll1 fused to the Fc portion of human IgG, was used to activate Notch signaling in the ECFC. An enzyme-linked immunoabsorbant assay was utilized to confirm that the concentration of ligand coated on the tissue culture plate surface correlated with the amount of ligand bound as previously described (Delaney, Varnum-Finney, Aoyama, Brashem-Stein, & Bernstein, 2005). The linear relationship between the amount of immobilized ligand and activation of Notch signaling was measured by the expression of the Notch downstream target gene Hey2 (Figure 2.2. a and b). After 3 days of culture, cells were harvested and the expression of Hey2 was examined by quantitative PCR. Expression of Hey2 increased as the concentration of Delta1$^{ext\text{-}IgG}$ increased, which indicated that human CB derived ECFCs were capable of responding to Notch ligand Dll1 stimulation.

We next evaluated whether Delta1$^{ext\text{-}IgG}$ influences other Notch related gene expression patterns in human CB ECFCs. Early passaged (2-4) ECFCs (N=5) were cultured in wells coated with Delta1$^{ext\text{-}IgG}$ at concentrations ranging from 0.312 to 20 μg/mL or with the same concentration of human IgG as the control

attachment ligand. Dll4, Notch 1, and Notch4 mRNA expression over 14 days of culture were unchanged in the ECFC treated with Delta1$^{ext\text{-}IgG}$ (Figure 2.3.). It is noteworthy that mRNA for the arterial-like endothelial gene markers Hey2 and EphrinB2 were significantly upregulated after 3 days of Delta1$^{ext\text{-}IgG}$ induction at the concentration of 10 μg/mL (Figure 2.2. b) while the expression of mRNAs for the venous-like endothelial gene markers CoupTFII and EphB4 remained at low levels of detection (Figure 2.2. b). Changes in Hey2 and CoupTFII gene expression at the transcript level upon Delta1$^{ext\text{-}IgG}$ binding were confirmed at the protein level (Figure 2.2. c). Immunoblot analysis indicated that the protein level changes of Hey2 and CoupTFII were consistent with their mRNA levels of change. These results demonstrated that Delta1$^{ext\text{-}IgG}$ was able to enhance Notch related gene expression in human CB ECFCs *in vitro*.

Next, we examined whether Delta1$^{ext\text{-}IgG}$ -induced EphrinB2 expression could be suppressed if Notch signaling was blocked via the use of a γ-secretase inhibitor L685458. The expression of Hes1 and Hey2 mRNA decreased with the addition of the inhibitor to the culture (Figure. 2.4. a and b). Interestingly, the Delta1$^{ext\text{-}IgG}$ -induced upregulation of mRNA for Hey2 and EphrinB2 was significantly decreased in the presence of 1μM L685458 for 3 days, whereas the expression of mRNA for CoupTFII was noticeably increased (Figure. 2.4. c). In addition, when control human CB ECFCs were treated with 1μM L685458 alone for 3 days, the expression of mRNAs for Hey2 and EphrinB2 were dramatically suppressed while the mRNA for CoupTFII was considerably enhanced when compared to

Delta$^{ext\text{-}IgG}$ treated cells (Figure. 2.4. c). Collectively, these observations demonstrated that immobilized Dll1 activated Notch signaling is able to regulate ECFC gene expression patterns *in vitro* over several days of exposure.

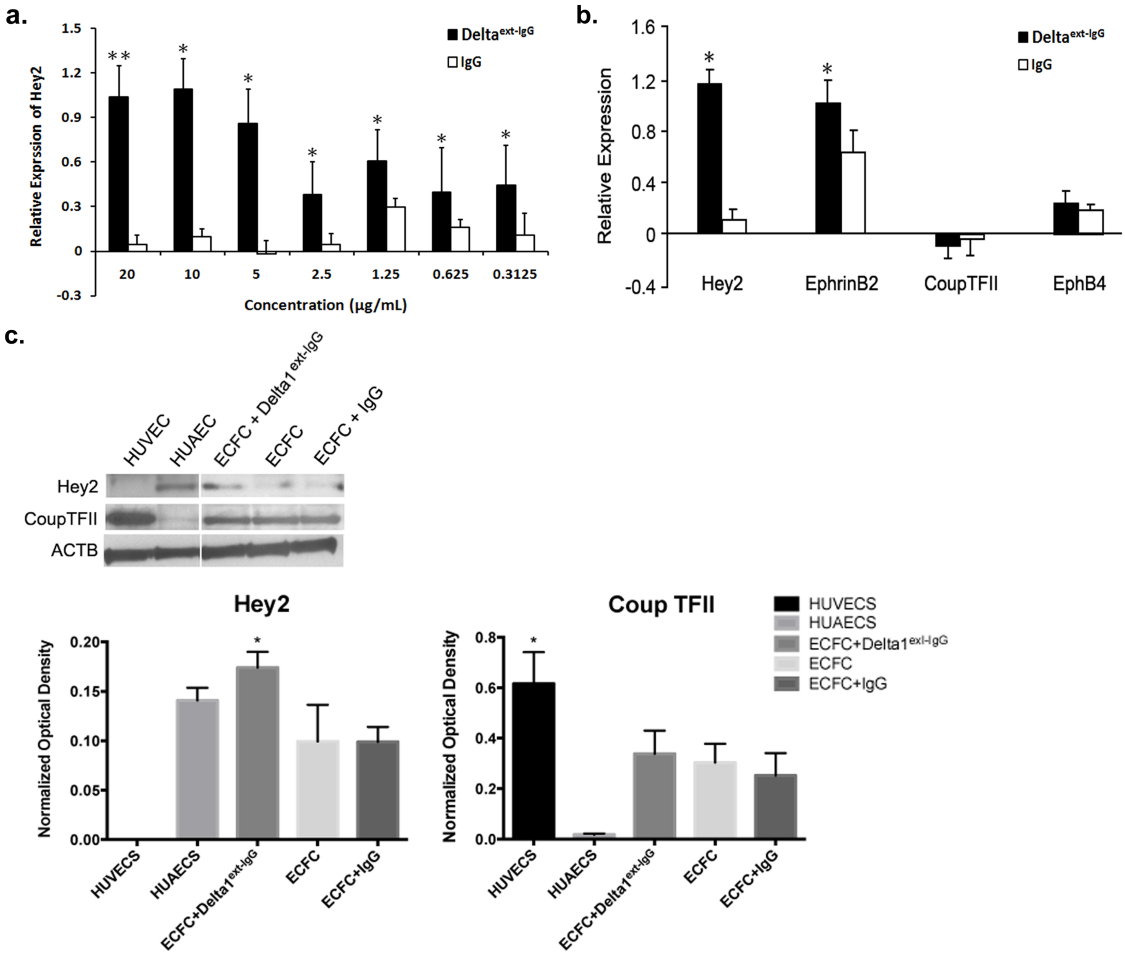

a.
Relative Exprssion of Hey2
Delta ext-IgG
IgG
Concentration (μg/mL)
20
10
5
2.5
1.25
0.625
0.3125
b.
Relative Expression
Hey2
EphrinB2
CoupTFII
EphB4
c.
HUVEC
HUAEC
ECFC + Delta1 ext-IgG
ECFC
ECFC + IgG
Hey2
CoupTFII
ACTB
Hey2
Normalized Optical Density
Coup TFII
HUVECS
HUAECS
ECFC+Delta1 ext-IgG
ECFC
ECFC+IgG

Figure 2.2. ECFCs respond to Notch signaling with increased expression of known downstream target genes. The dose-dependent activation of endogenous Notch signaling in human cord blood derived ECFCs is indicated by Hey2 (a) expression after 3 days of incubation in various concentrations of $Delta^{1ext\text{-}IgG}$. (b) Quantitative RT-PCR analysis revealed Hey2 and EphrinB2 transcripts were significantly increased after 3 days of stimulation with 10 μg/mL $Delta1^{ext\text{-}IgG}$, whereas the expression of CoupTFII and EphB4 were not significantly affected. Expression levels were presented as a fold change (in ^{2}logarithmic scale) compared to baseline levels and were normalized by using ATP5B as a housekeeping gene. The expression at day 0 in nontreated cells serveed as a baseline value. N=5. *P<0.05. (c) Upper panel shows representative immunoblots of Hey2 and CoupTFII proteins and lower panel shows quantification (protein expression levels were normalized by using β-actin). N=3. *P<0.05. Reprinted with permission from "Notch ligand Delta-like 1 promotes *in vivo* vasculogenesis in human cord blood-derived endothelial colony forming cells" by Kim et al., 2015, Cytotherapy, 17(5):579-92. Figure 2.2. was from combined effort of my co-worker, Lan Huang and me (H. Kim, Huang, et al., 2015).

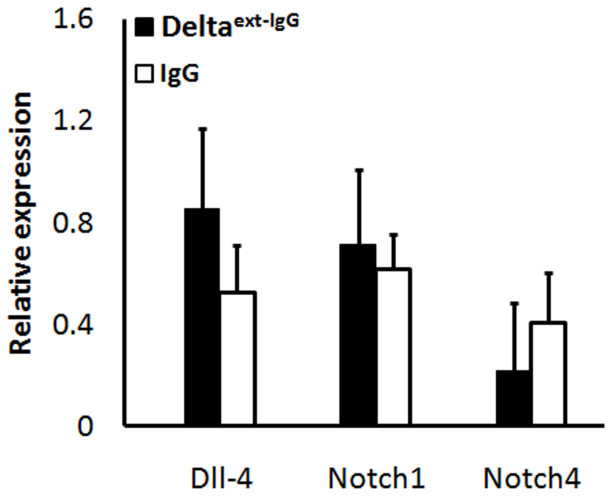
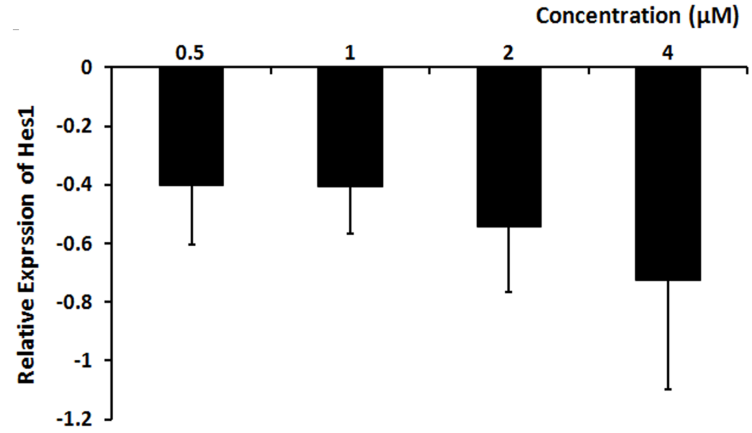
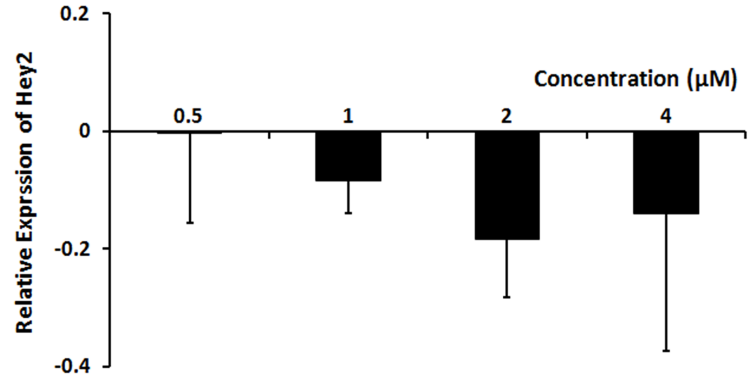


Figure 2.3. Alteration of Notch gene expression in ECFC response to 3 days of Delta1$^{ext\text{-}IgG}$ stimulation. Quantitative PCR analysis reveals that expression of mRNA levels for Dll4, Notch1, and Notch4 was unchanged upon stimulation of ECFC upon co-culture with Delta1$^{ext\text{-}IgG}$ over 3 days. Expression levels are presented as a fold change (in ^{2}logarithmic scale) compared to baseline levels, which were normalized using ATP5B as a housekeeping gene. The expression at Day 0 in non-treated cells acts as baseline. N=5. *P<0.05. Reprinted with permission from "Notch ligand Delta-like 1 promotes *in vivo* vasculogenesis in human cord blood-derived endothelial colony forming cells" by Kim et al., 2015, Cytotherapy, 17(5):579-92. Figure 2.3. was from combined effort of my co-worker, Lan Huang and me (H. Kim, Huang, et al., 2015).

a.


Concentration (μM)
0.5
1
2
4
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
Relative Exprssion of Hes1

b.


0.2
Concentration (μM)
0.5
1
2
4
0
-0.2
-0.4
Relative Exprssion of Hey2

c.

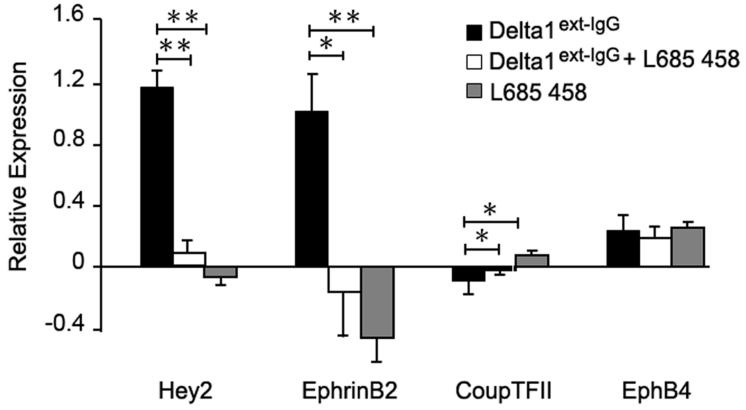

1.6
1.2
0.8
0.4
0
-0.4
Relative Expression
**
**
**
*
*
*
Delta1 ext-IgG
Delta1 ext-IgG + L685 458
L685 458
Hey2
EphrinB2
CoupTFII
EphB4

Figure 2.4. The effect of γ-secretase inhibitor L685458 on Notch signaling. The dose-dependent inhibition of endogenous Notch signaling in human CB derived ECFCs is indicated by Hes1 (a) and Hey2 (b) expression after 3 days of incubation in various concentrations of L685458 using quantitative PCR. Expression levels are presented as a fold change (in ^{2}logarithmic scale) compared to baseline levels, which were normalized using ATP5B as a housekeeping gene. The expression at Day 0 in non-treated cells acts as baseline. N=5. (c) Quantitative RT-PCR analysis revealed that Delta1$^{ext\text{-}IgG}$-induced upregulation of Hey2 and EphrinB2 was dramatically attenuated in the presence of 1μM γ-secretase inhibitor L685458 while CoupTFII was significantly upregulated. Expression levels are presented as a fold change (in ^{2}logarithmic scale) compared to baseline levels and were normalized by using ATP5B as a housekeeping gene. The expression at day 0 in non-treated cells serves as a baseline. N=5. *P<0.05, or **P<0.01. Reprinted with permission from "Notch ligand Delta-like 1 promotes *in vivo* vasculogenesis in human cord blood-derived endothelial colony forming cells" by Kim et al., 2015, Cytotherapy, 17(5):579-92. Figure 2.4. was from combined effort of my co-worker, Lan Huang and me (H. Kim, Huang, et al., 2015).

Dll1 expression by OP9 stromal cells significantly enhances *in vivo* postnatal vasculogenesis

We subsequently tested whether human CB ECFCs exposed to immobilized Dll1 (Delta1$^{ext\text{-}IgG}$) would increase vasculogenesis after they were implanted *in vivo*. ECFCs were treated with 10 μg/mL of Delta1$^{ext\text{-}IgG}$ for 3 days *in vitro*, then suspended in a collagen-fibronectin matrix and subcutaneously implanted into immunodeficient mice as described (Yoder et al., 2007). After 14 days, the mice were euthanized, the grafts were harvested, and implants were analyzed for blood vessel formation (Figure 2.5. a). Our data indicated that *in vitro* Delta1$^{ext\text{-}IgG}$-primed human CB ECFCs were unable to enhance vessel formation *in vivo*, since the total vascular area of the human vessels was unchanged compared to IgG control cultured cells (Figure 2.5. a and b). These data indicate that preconditioning of ECFC *in vitro* with Notch activation fails to translate into enhanced vasculogenic properties in ECFC *in vivo*.

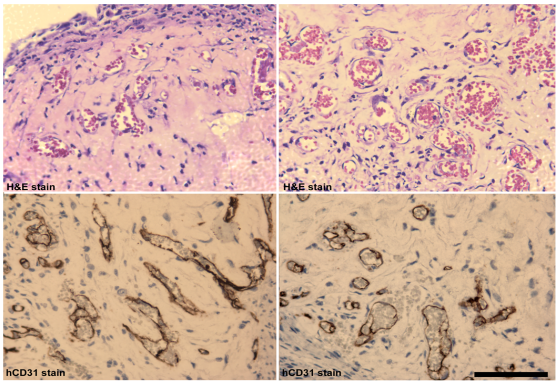
Knowing that activation of Notch signaling is mediated by cell-cell interaction between Notch ligands and receptors *in vitro* and *in vivo* (Kume, 2009), we hypothesized that co-implantation of ECFCs with stromal cells constitutively expressing Dll1 may be required to enhance vasculogenesis *in vivo*. ECFCs were cultured in standard conditions and then co-implanted with OP9-Delta-like 1 stromal cells (OP9-DL1) into immunodeficient mice. After 14 days, the mice were euthanized; the grafts were harvested and investigated for human blood vessel formation. Use of a specific anti-human CD31 antibody revealed that human CB

ECFCs were able to form microvessels perfused with murine red blood cells in the grafts (Figure 2.6. a). Of interest, the vessel number and morphology differed between the collagen matrices containing ECFC co-implanted with OP9-DL1 (ECFC/OP9-DL1) cells and the control implants (the collagen matrices containing ECFC alone or ECFC with OP9 (ECFC/OP9)). The quantification of murine erythrocyte-containing human microvessels (Figure 2.6. b) revealed a significantly greater number of $hCD31^+$ vessels were present in ECFC/OP9-DL1 implants compared to the controls (ECFC alone vs. ECFC/OP9 vs. ECFC/OP9-DL1 was 30.03 ± 8.14 vs. 53.32 ± 7.19 vs. 129.10 ± 27.80 vessels/mm^2, respectively, $P<0.05$). In addition, the size distribution of $hCD31^+$ microvessels was altered with the ECFC/OP9-DL1 implants compared to the control implants (Figure 2.6. c). Noticeably, upon Dll1 stimulation human cord blood ECFCs gave rise to significantly more large area microvessels (1000-4000 μm^2) and less small ones (51-100 μm^2) (Figure 2.6. c). Furthermore, the average vessel area was distinctly increased with the ECFC/OP9-DL1 implants compared to the control implants (Figure 3.6. d, ECFC alone vs. ECFC/OP9 vs. ECFC/OP9-DL1 was 223.81 ± 15.47 vs. 266.03 ± 39.28 vs. 384.61 ± 42.73 μm^2, respectively, $P<0.05$). These alterations resulted in the overall $hCD31^+$ vascular area (=average vessel density x average vessel area) being significantly enhanced in the ECFC/OP9-DL1 implants compared to the control implants (Figure 2.6. e).

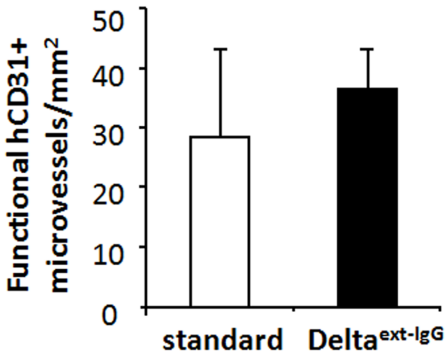
Notch1 and Notch2 protein levels were significantly upregulated in implants with OP9-DL1, but Notch4 expression level was distinctly decreased (Figure 2.6. f). In

addition, stimulation with the OP9-DL1 Notch ligand has been reported to cause rapid nuclear translocation of the cleaved domain of Notch1 (Krebs et al., 2000). Consistently, Notch1 activation was widely detected in nuclei of human ECFC-derived vessels (in implants containing OP9-DL1) as shown by specific EC nuclear immunostaining for cleaved Notch1 (Val1744) (Figure 2.6. g). This observation indicated that human ECFC-derived vessels were responsive to Dll1 ligand presentation *in vivo* and Notch1 signaling was persistently activated in ECFC/OP9-DL1 implants *in vivo*. Also, anti-mouse smooth muscle α actin (αSMA) antibody stain (red) was detected in peri-vascular cells located around human ECFC-derived $CD31^+$ (brown) microvessels in co-implants with OP9-DL1 (Figure 2.6. h). Together, these data suggest that Dll1 binding to Notch1 significantly modulates vasculogenesis *in vivo* by inducing Notch signaling and promoting more ECFC-derived vessel formation with an overall enlarged vascular area and recruitment of host murine peri-vascular cells to the human vessels.

a.


H&E stain
H&E stain
hCD31 stain
hCD31 stain
standard
Delta^ext-IgG

b.


Functional hCD31+ microvessels/mm²
50
40
30
20
10
0
standard
Delta^ext-IgG

c.

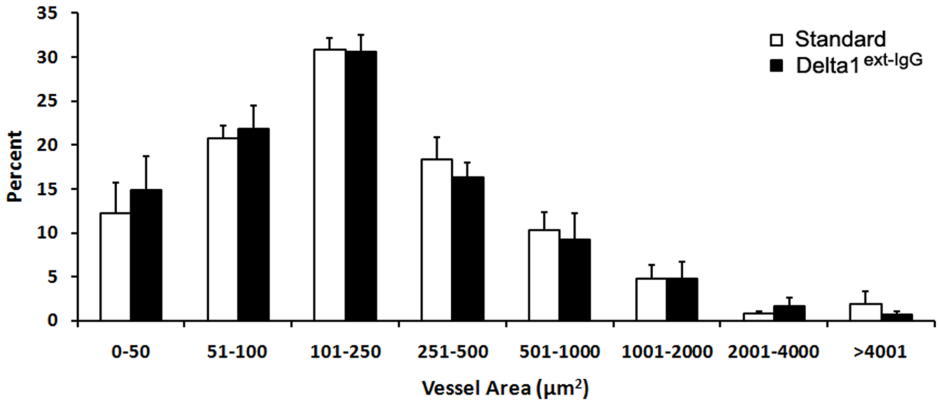
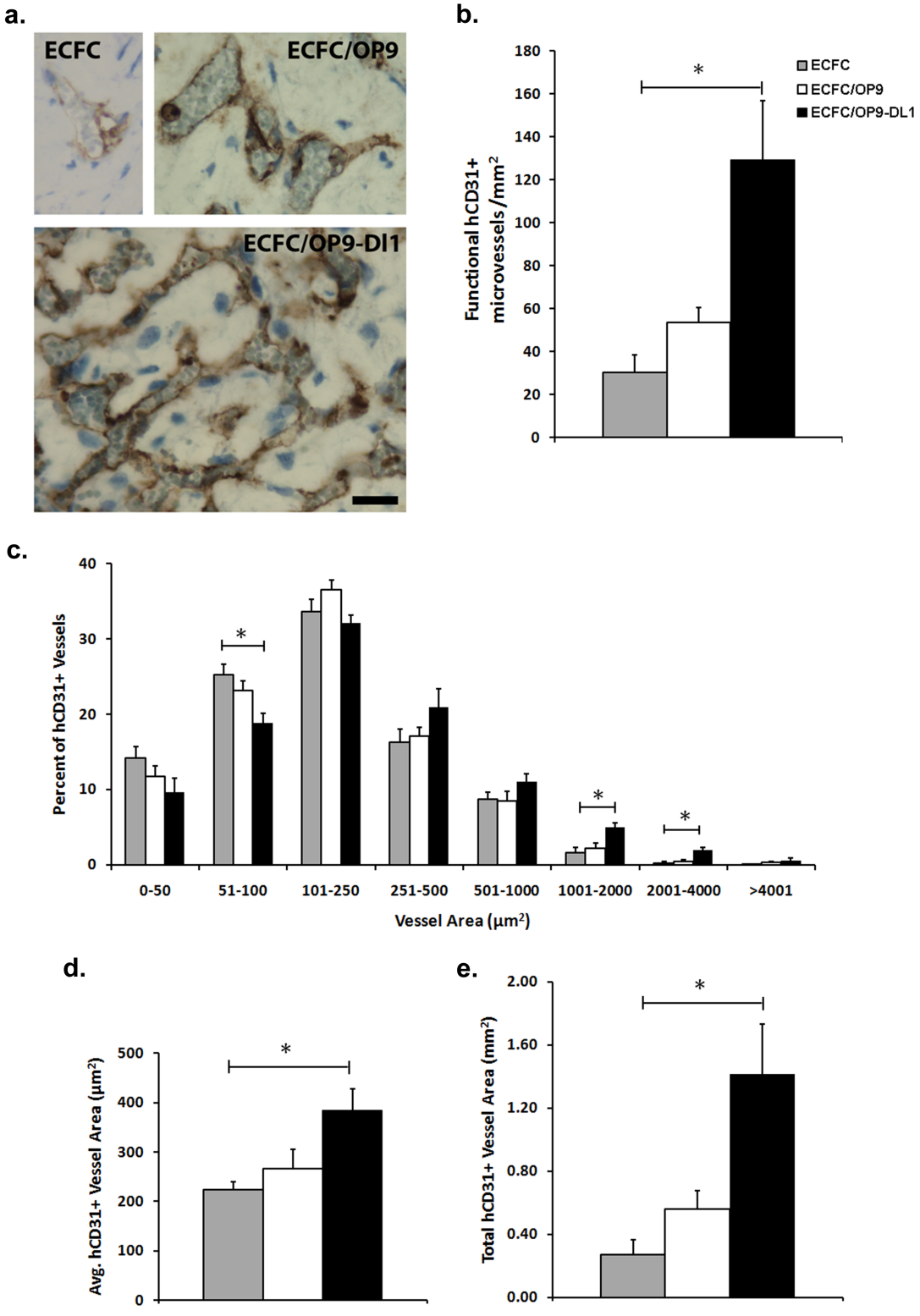
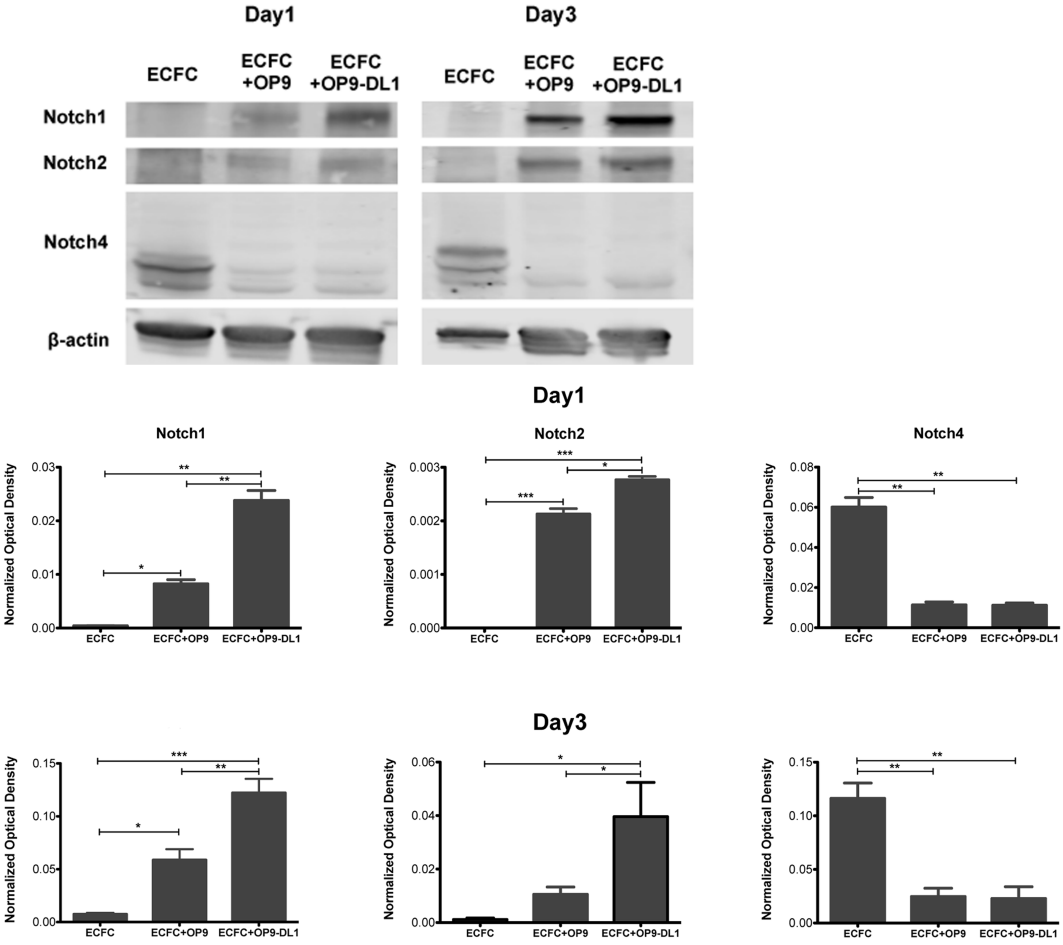

Standard
Delta1 ext-IgG
Percent
35
30
25
20
15
10
5
0
0-50
51-100
101-250
251-500
501-1000
1001-2000
2001-4000
>4001
Vessel Area (µm²)

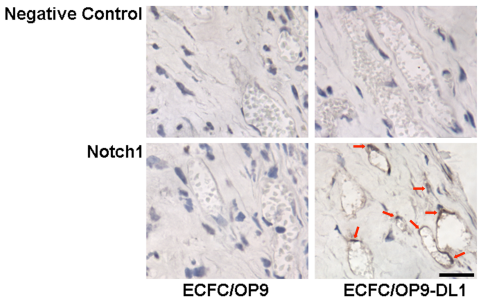
Figure 2.5. *In vitro* Delta1$^{ext\text{-}IgG}$-primed ECFCs fail to enhance vasculogenesis *in vivo*. (a) H&E staining indicates microvessel formation in collagen-fibronectin matrices after 14 days of implantation. Anti-human CD31 staining further confirms the human origin of these vessels. Results suggest that human cord blood derived ECFCs cultured in the presence of Delta1$^{ext\text{-}IgG}$ retain vessel-forming ability *in vivo*. Moreover, the number of vessels formed by human cord blood derived ECFCs and perfused with murine red blood cells per mm^2 in the matrix demonstrate no observable difference between Delta1$^{ext\text{-}IgG}$ treated and nontreated samples (b). In addition, the size distribution of both populations of microvessels is similar (c). N=6. Reprinted with permission from "Notch ligand Delta-like 1 promotes *in vivo* vasculogenesis in human cord blood-derived endothelial colony forming cells" by Kim et al., 2015, Cytotherapy, 17(5):579-92. Figure 2.5. was from combined effort of my co-worker, Lan Huang and me (H. Kim, Huang, et al., 2015).


a.
ECFC
ECFC/OP9
ECFC/OP9-Dl1
b.
ECFC
ECFC/OP9
ECFC/OP9-DL1
Functional hCD31+ microvessels/mm2
180
160
140
120
100
80
60
40
20
0
*
c.
Percent of hCD31+ Vessels
40
30
20
10
0
0-50
51-100
101-250
251-500
501-1000
1001-2000
2001-4000
>4001
Vessel Area (µm2)
*
*
*
d.
Avg. hCD31+ Vessel Area (µm2)
500
400
300
200
100
0
*
e.
Total hCD31+ Vessel Area (mm2)
2.00
1.60
1.20
0.80
0.40
0.00
*

f.


Day1
Day3
ECFC
ECFC +OP9
ECFC +OP9-DL1
Notch1
Notch2
Notch4
β-actin
Day1
Notch1
Notch2
Notch4
Normalized Optical Density
ECFC
ECFC+OP9
ECFC+OP9-DL1
Day3

g.


Negative Control
Notch1
ECFC/OP9
ECFC/OP9-DL1

h.

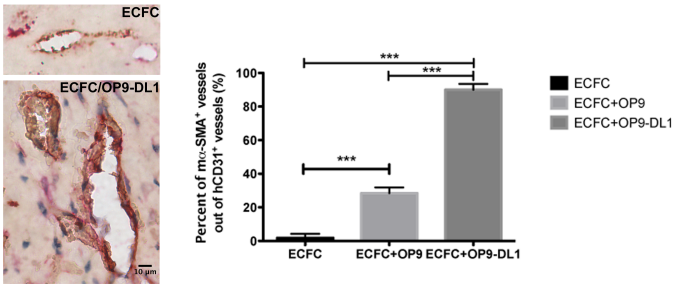

ECFC
ECFC/OP9-DL1
10 μm
Percent of mα-SMA+ vessels out of hCD31+ vessels (%)
ECFC
ECFC+OP9
ECFC+OP9-DL1

Figure 2.6. *In vivo* Dll1 stimulation boosts the formation of functional vessels. (a) Anti-human CD31 staining identified human cord blood derived ECFCs alone or combined with OP9 or OP9-DL1, that have formed microvessels in collagen matrices after 14 days of implantation. Upon the stimulation of Dll1, there was a significant increase in the number of vessels formed by human cord blood derived ECFCs and perfused with murine red blood cells per mm^2 in the matrix (b). In addition, the size distribution of $hCD31^+$ microvessels was noticeably altered (c), shifting towards larger sized vessels (1001-4000 μm^2) with Dll1 stimulation. Moreover, vessel morphology was significantly altered by the presence of Dll1 with increased average vessel area (d) and total vascular areas (d). (f) Upper panel shows representative immunoblots of Notch1, Notch2, and Notch4 proteins and lower two panels show quantification of repeated experiments (protein expression levels were normalized using β-actin). N=3. *P<0.05. Anti-cleaved Notch1 (Val1744) antibody staining (g) in endothelial nuclei confirms that the activation of Notch1 is detected *in vivo* in newly-formed human vessels in the presence of Dll1. N=6. Scale bar represents 100 μm. (h) Anti-mouse smooth muscle α actin (αSMA) antibody staining (red) was detected in peri-vascular cells around human ECFC-derived $CD31^+$ vessels (brown) in implants with OP9-DL1 stromal cells. N=3. Scale bar represents 10 μm. *P<0.05. Reprinted with permission from "Notch ligand Delta-like 1 promotes *in vivo* vasculogenesis in human cord blood-derived endothelial colony forming cells" by Kim et al., 2015, Cytotherapy, 17(5):579-92. Figure 2.6. (a-e) were performed by

co-worker, Lan Huang. Figure 2.6. (f-h) were from my original work (H. Kim, Huang, et al., 2015).

Dll1 expressed by OP9 stromal cells significantly reduces apoptosis of human cord blood ECFCs in three dimensional (3D) collagen matrices.

To begin to understand the mechanisms through which Notch activation could enhance vasculogenesis of the human ECFC, we tested whether the presence of OP9-DL1 decreases apoptosis of human CB ECFCs in the 3D collagen implants. After one, two, three, or five days, the cells were recovered and assessed for apoptosis of ECFCs using Annexin V and propidium iodide staining. Implanted ECFCs started undergoing apoptosis on day 1 and a significant majority of the cells died on day 2 and 3 after implantation (Figure 2.7. a). ECFC co-implanted with OP9-DL1 (1:1 cell ratio) revealed a significantly higher percentage of the Annexin V^-/PI^- viable cell population compared to ECFC alone or ECFC with OP9 implantation on day 1 and 3 (Figure 2.7. b and c). These results indicated that co-implantation of ECFC with OP9-DL1 stromal cells increased survival of ECFC within the 3D-collagen matrices. Since caspase activation is a hallmark of apoptosis, we examined ECFC for evidence of caspase activation within the 3D matrices. The caspase assay we employed allowed detecting fluorescein-labeled inhibitors that bind to activated caspase-1 and -3/7 within cells. The inhibitors bind with 1:1 stoichiometry to the active centers of activated caspases. Thus, the assay can be used for quantitative analysis of the activated caspases in the cells. In every experiment performed, the caspase-$3/7^-/PI^-$ cell subset of ECFC co-implanted with OP9-DL1 was significantly higher on day 3 compared to implanted ECFC alone or ECFCs with OP9 implantation (Figure 2.7. d). ECFC co-implanted with OP9-DL1 also displayed a greater percentage of caspase-1^-

/PI$^-$ cells compared to ECFC alone or ECFC with OP9 implantation on day 2 and 3 (Figure 2.7. e). These observations indicate that Notch activation by OP9-DL1 stromal cells reduced activated caspase-3/7 and caspase-1 within ECFCs in collagen matrices. Therefore, we conclude that Notch activation of ECFC in collagen matrix implants enhanced survival of ECFCs by reducing activation of caspase-3/7 and caspase-1.

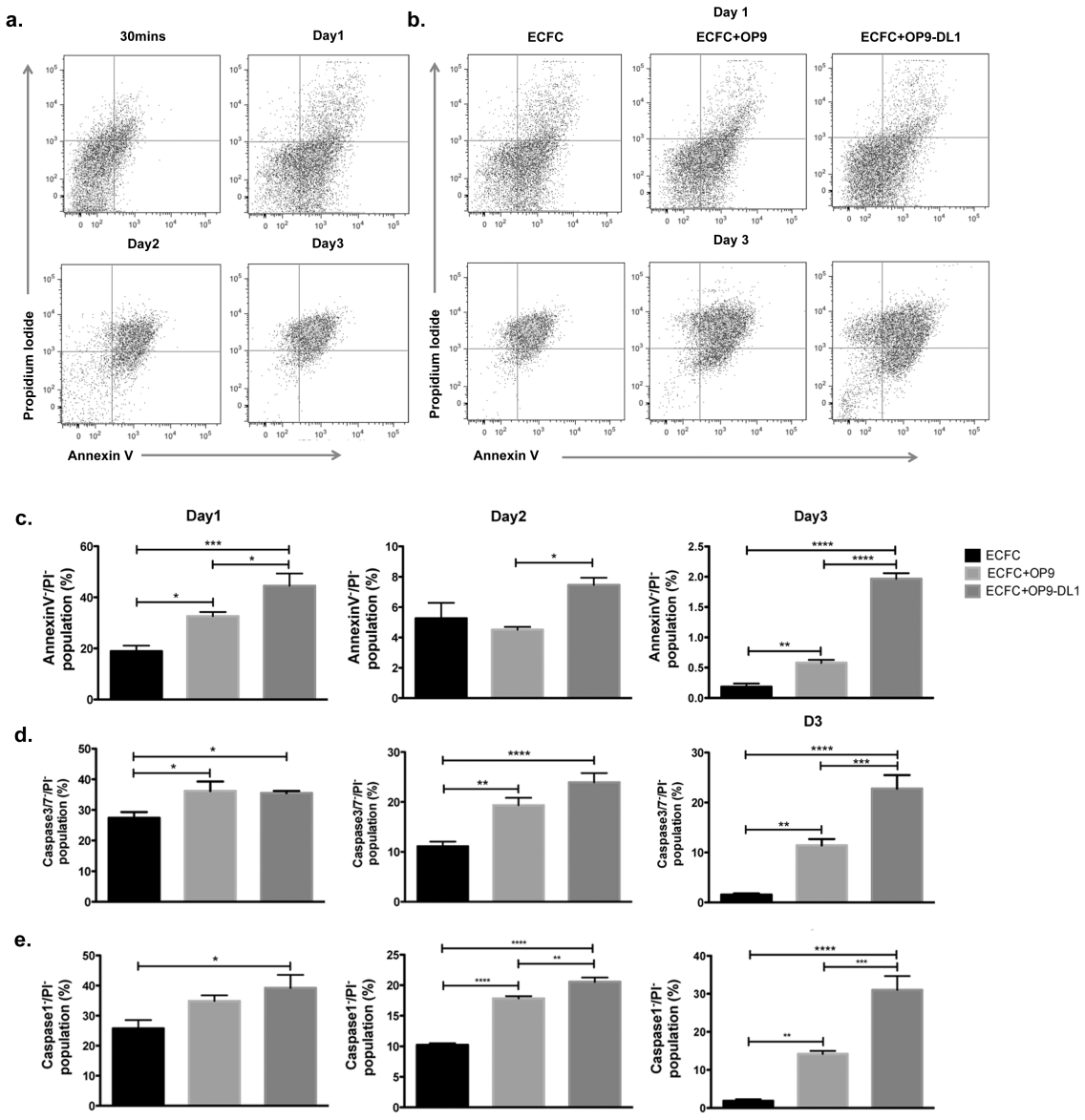

a.
30mins
Day1
Day2
Day3
Propidium Iodide
Annexin V
b.
Day 1
ECFC
ECFC+OP9
ECFC+OP9-DL1
Day 3
Propidium Iodide
Annexin V
c.
Day1
Day2
Day3
AnnexinV-/PI- population (%)
ECFC
ECFC+OP9
ECFC+OP9-DL1
d.
D3
Caspase3/7-/PI- population (%)
e.
Caspase1-/PI- population (%)

Figure 2.7. OP9-DL1 stimulation diminishes apoptosis of ECFCs in 3D collagen matrices. (a) Representative dot plots of apoptosis analysis of ECFCs at 30 minutes or on day 1-3. (b) Representative dot plots of apoptosis analysis of ECFC alone, co-implanted with OP9 or OP9-DL1 on day 1-3. (c) The Annexin V^- /PI^- viable population of ECFC co-implanted with OP9-DL1 was significantly higher on day 1 and 3 compared to implantation of ECFC alone or ECFC with OP9 on day 1 and 3. (d) The percentage of the Caspase-3/7^-/PI^- population of ECFC co-implanted with OP9-DL1 was significantly greater compared to ECFC alone or with OP9 on day 3. (e) ECFC co-implanted with OP9-DL1 displayed a higher percentage of Caspase-1^-/PI^- cells compared to ECFC alone or ECFC with OP9 co-implants. N=6. *P<0.05. Reprinted with permission from "Notch ligand Delta-like 1 promotes *in vivo* vasculogenesis in human cord blood-derived endothelial colony forming cells" by Kim et al., 2015, Cytotherapy, 17(5):579-92. Figure 2.7. was from my original work (H. Kim, Huang, et al., 2015).

2.4. Discussion

Vasculogenesis is the process of primary capillary plexus formation from angioblasts during embryogenesis. Remodeling of the capillary plexus normally occurs via angiogenesis and arteriogenesis to form the hierarchical mature systemic vasculature (Carmeliet, 2000; Hanahan, 1997). It has been well established that Notch signaling plays an important role in regulating vascular patterning and remodeling during development (Benedito et al., 2008; Copeland, Feng, Neradugomma, Fields, & Vivian, 2011; Y. H. Kim et al., 2008; Krebs, Starling, Chervonsky, & Gridley, 2010; Trindade et al., 2008; Uyttendaele, Ho, Rossant, & Kitajewski, 2001) and after birth (Limbourg et al., 2007; Takeshita et al., 2007). Previous studies in murine embryonic development showed that ectopic Notch1 activation in ECs caused vascular remodeling defects with enlarged vessel caliber (Copeland et al., 2011; Krebs et al., 2010). In contrast, small sized vessels were present upon loss of active Notch signaling (Benedito et al., 2008; Copeland et al., 2011). We report that the *in vivo* presence of stromal cell associated Notch ligand Dll1 led to enhanced vasculogenesis and vascular remodeling in human cord blood ECFCs, characterized by increased vessel density and enlarged vessel area (Figure 2.6.). Accordingly, our observations coincide with the previous studies indicating that Dll1-dependent Notch1 signaling regulates vessel size and state in murine embryogenesis.

A role for mesenchymal stromal cells (MSC) in promoting enhanced human ECFC vasculogenesis *in vivo* has been reported by several laboratories (P. Allen,

K.-T. Kang, & J. Bischoff, 2013; Au, Daheron, et al., 2008; Au, Tam, Fukumura, & Jain, 2008; Kang, Allen, & Bischoff, 2011; Lin, Moreno-Luna, Zhou, Pu, & Melero-Martin, 2012; Melero-Martin et al., 2008; Traktuev et al., 2009; X. Wu et al., 2004). Indeed, conditioned medium secreted by multiple tissue-derived cultured MSCs promotes ECFC vasculogenesis *in vivo*, although the specific molecules promoting enhanced ECFC function remain elusive (Lin et al., 2012). Surprisingly, we did not find a significant improvement in ECFC vasculogenic properties when the murine OP9 MSCs were co-implanted with the ECFC in our studies. We speculate that differences in the types and composition of the 3D scaffolding proteins and strain of immunodeficient mice may play a role in the extent to which MSC promote human ECFC vasculogenesis *in vivo*. While there are numerous matrices that permit ECFC and MSC, but not ECFC alone, to form vessels *in vivo* upon implantation into nude mice (Patrick Allen et al., 2013; Allen, Melero-Martin, & Bischoff, 2011), cord blood ECFC alone form human blood vessels in tissue engineered skin substitutes implanted in C.B-17 severe combined immunodeficient (SCID)/beige mice (Benjamin R. Shepherd et al., 2006) or in type I collagen matrices implanted in non-obese diabetic (NOD)/(SCID) mice (L. Huang et al., 2011; Yoder et al., 2007). Further work to directly compare ECFC vasculogenesis in various matrices in several different immunodeficient murine hosts will be required to address the discrepancies in the literature.

VEGF plays a critical role in determining arterial-venous specification and the concentration of VEGF influences this determination (Lanner, Sohl, & Farnebo, 2007). In cultured murine embryonic stem cells (ESCs), a high concentration of VEGF (50 ng/mL) drove an arterial-like endothelial gene expression pattern while a low dose encouraged venous-like endothelial gene expression (Lanner et al., 2007). Similarly, addition of 10 ng/mL of rhVEGF-A to cultured cells upregulated an arterial-like endothelial gene expression pattern in hMAPCs and in hMSCs (Aranguren et al., 2007; Zhang et al., 2008). Recently, in the presence of 50 ng/mL of rhVEGF-A, arterial-like endothelial genes and downstream genes of Notch signaling were increased in cultured ECFCs (Boyer-Di Ponio et al., 2014). The arterial-like endothelial genes induced by VEGF were decreased with treatment using γ-secretase inhibitor on cultured ECFCs (Boyer-Di Ponio et al., 2014). This study indicated that VEGF-dependent induction leads to arterial-like gene expression with Notch signaling activation. Our results consistently confirmed prior work that rhVEGF-A at 50 ng/mL was sufficient to enhance expression of the arterial-like endothelial genes with activation of Notch in cultured CB ECFC. In addition, Notch stimulated preconditioning *in vitro* was able to induce an arterial-like gene expression pattern *in vitro*. However, preconditioning of ECFC *in vitro* with Notch stimulation failed to promote an arterial-like phenotype *in vivo*, suggesting that *in vitro* priming is not sufficient for *in vivo* specification in our experimental model. Of interest, *in vivo* co-implantation of OP9-DL1 stromal cells with ECFCs significantly enhanced vasculogenesis *in vivo* by promoting more ECFC-derived vessel formation with

an overall enlarged vascular area and recruiting of murine peri-vascular cells around ECFC-derived vessels (Figure 2.6.). Shepherd et al. (B. R. Shepherd, Jay, Saltzman, Tellides, & Pober, 2009) reported that co-implantation of human aortic smooth muscle cells promoted larger vessel size with peri-endothelial investment at 60 days post-implantation. While we implanted the OP9-DL1 stromal cells with the ECFC, we observed an effect of Notch activation on human vessel size and recruitment of host peri-vascular cells. Future studies utilizing specific perivascular cell subsets may indicate whether there are cell specific influences provided by the mesenchymal cells on the human ECFC.

In the present work, implanted human CB ECFCs started undergoing apoptosis on day 1. After 2-5 days of implantation, most implanted ECFCs died with only 5-8% of total implanted cells persisting as perfused vessels. Previous studies have indicated that human ECFC cell-lined vessels formed within subcutaneous implants in mice were found as early as day 1 or 2 following implantation, but these lumenized structures lacked significant perfusion with murine red blood cells at those early time points. However, ECFC derived vessels perfused with host murine red blood cells (perfusion also confirmed by human specific lectin infusion and contrast-enhanced ultrasound flow detection) were detected after 3-4 days of implantation *in vivo* (Patrick Allen et al., 2013). Thus, implanted endothelial cells had to survive at least 3-4 days *in vivo* to be able to contribute to a stable perfused capillary network. Given the proclivity of capillary networks to destabilize and regress with endothelial apoptosis in the absence of peri-

endothelial support cells or adhesion of the endothelial cells to matrix proteins via cell surface integrin receptors (Brooks et al., 1994; Fukai et al., 1998; Pollman, Naumovski, & Gibbons, 1999; Stratman, Schwindt, Malotte, & Davis, 2010), understanding the importance of a balance of pro-apoptotic versus anti-apoptotic signals in the plasticity of the capillary plexus has become an important paradigm. Peri-vascular support cells can induce endothelial cells to secrete basement membrane proteins that enhance endothelial cell integrin mediated attachment and increases in Bcl-2 expression to protect cells from apoptosis (Pollman et al., 1999; Stromblad, Becker, Yebra, Brooks, & Cheresh, 1996). While human umbilical vein endothelial cells (HUVECs) undergo apoptosis when implanted in immunodeficient mice within 24h, Bcl-2 overexpression in HUVECs rescues them from apoptotic death and enhances the number and complexity of human vessels formed (Zheng et al., 2000). Bcl-2 overexpression in HUVECs also increases the number of human and mouse endothelial cell-lined vessels (vascularization) in implants and induces maturation of vessels by increasing tissue perfusion (Enis et al., 2005; Benjamin R. Shepherd et al., 2006). The frequency of the Annexin V^-/PI^- viable cell population and overall $hCD31^+$ vascular area in the ECFC/OP9-DL1 implants reflected a four-fold increase compared to the ECFC/OP9 implants (Figure. 2.6. e and 2.7. c), suggesting a direct effect between diminishing apoptosis and increased vessel formation by Notch activation in ECFC *in vivo*. Our results suggest that improvement of cell survival of ECFCs by Dll1-dependent Notch1 signaling activation (Figure. 2.6. f)

leads to vessel stabilization and promotes functional vessel formation in 3D collagen matrices.

In summary, we have demonstrated that human cord blood ECFCs robustly form microvessels exhibiting a distinct remodeling pattern with increased vessel number and enlarged vessels by diminishing apoptosis of CB ECFCs during OP9-DL1 mediated Notch activation. While other studies have shown that stromal cell co-implantation with ECFCs increases vessel number and maturation *in vivo* (Melero-Martin et al., 2010; Melero-Martin et al., 2008), we did not observed any enhancement of vasculogenesis by co-implanting ECFCs with OP9 stromal cells. However, co-implantation of OP9-DL1 led to constitutive ECFC Notch activation and resulted in greater numbers and maturation of human blood vessels *in vivo*. Further studies to present Notch activating ligands embedded within the matrix molecules may permit the modulation of human vessel formation using ECFC alone.

CHAPTER 3. HUMAN PLATELET LYSATE IMPROVES HUMAN CORD BLOOD DERIVED ECFC SURVIVAL AND VASCULOGENESIS IN THREE DIMENSIONAL (3D) COLLAGEN MATRICES

The following chapter is reprinted with permission from “Human platelet lysate improves human cord blood derived ECFC survival and vasculogenesis in three dimensional (3D) collagen matrices” by Kim et al., 2015, Microvascular Research, 101:72-81 (H. Kim, Prasain, et al., 2015).

3.1. Introduction

The progenitor cells for the endothelial lineage play critical roles in vascular homeostasis and regeneration in adult subjects (Asahara et al., 1997; Hirschi et al., 2008). Endothelial colony forming cells (ECFC) are derived from a rare circulating subset that may arise from resident endothelium of established blood vessels in man (Hirschi et al., 2008). We have successfully isolated human peripheral blood (PB) or umbilical cord blood (CB) derived circulating ECFC that display a hierarchy of proliferative potential through the use of single cell clonogenic and functional assays (L. Huang et al., 2011; Ingram et al., 2004). Human CB derived ECFC also display *de novo* vessel forming ability in nude or non-obese diabetic/severe combined immunodeficient mice (NOD/SCID) *in vivo* after subcutaneous implantation of ECFC in type I collagen matrices and upon perfusion with host murine vessels, become a part of the systemic host circulation (Au, Daheron, et al., 2008; Cheng et al., 2011; Critser, Kreger, Voytik-

Harbin, & Yoder, 2010; L. Huang et al., 2011; Melero-Martin et al., 2008; Melero-Martin et al., 2007; Reinisch et al., 2009; Whittington, Yoder, & Voytik-Harbin, 2013; Yoder et al., 2007).

Previous studies have reported that human ECFC-derived vessels formed in subcutaneous implants were found as early as day 1 or 2 after implantation in NOD/SCID mice (P. Allen et al., 2013). However, these premature vessels were not properly perfused with murine red blood cells of the host circulatory system at those early time points. Proof of human ECFC cell-lined vessel connection to host circulation system after 3-4 days of implantation *in vivo* was subsequently reported (P. Allen et al., 2013). It has been reported that up to 60% of human umbilical vein endothelial cells (HUVECs) undergo apoptosis in the first 24 hours after suspension in collagen gels and there is further loss of vascular structures in 3D type I collagen matrices after 24 hours of culture (Ilan et al., 1998). In our previous study, we demonstrated that implanted human CB ECFC containing collagen matrices displayed ECFC undergoing apoptosis on day 1 following implantation (H. Kim, Huang, et al., 2015). After 2-3 days of implantation, most implanted ECFC died with only 1-3% of the total implanted cells demonstrating viability in the subcutaneous implants (H. Kim, Huang, et al., 2015). Since the stability of newly formed vessels requires systemic blood flow, theses data suggest that implanted endothelial cells must survive at least 3-4 days *in vivo* to form a stable perfused capillary network connected to the host circulatory system.

The microvascular network has the intrinsic capacity to remodel itself through changes in endothelial cell differentiation, growth, migration, and matrix modification (Hanahan & Folkman, 1996; Risau, 1995). Previous studies have reported that cytokines, matrix proteins, and integrins are important factors in capillary endothelial network formation and remodeling in various matrix models (Matrigel and type I collagen matrix systems) (Critser et al., 2010; Fukai et al., 1998; Hynes, 1992; Ranta, Mikkola, Ylikorkala, Viinikka, & Orpana, 1998; Sacharidou, Stratman, & Davis, 2012; Stratman, Davis, & Davis, 2011; Whittington et al., 2013). The absence of peri-endothelial support cells or lack of endothelial cell adhesion to matrix proteins causes destabilization and regression of capillary structures within Matrigel or 3D type I collagen matrices (Brooks et al., 1994; Fukai et al., 1998; Pollman et al., 1999; Stratman et al., 2010). Apoptosis of endothelial cells has been observed in the context of dynamic capillary network remodeling in the various matrix models both *in vitro* and *in vivo* and is thought to play a necessary role for optimal network formation (Brooks et al., 1994; Fukai et al., 1998; Pollman et al., 1999; Stratman et al., 2010). On the other hand, the generation of anti-apoptotic signals is also required to maintain integrity of the vascular network following realignment of certain matrix proteins and cell-cell or cell-matrix interactions during the process (Pollman et al., 1999; Shih et al., 2011). Thus, understanding the balance between pro-apoptotic and pro-survival signals is a critical point in capillary structure formation and the remodeling process.

Apoptosis is a cellular process in tissue development that generally culminates with the sequential activation of caspases, the cysteine proteases for cleavage of proteins (Duprez et al., 2009; Sprick & Walczak, 2004; Taylor et al., 2008). There are two pathways that result in apoptosis depending on the apoptotic signal. The extrinsic pathway is initiated by ligand binding to membrane receptors of the death receptor family, whereas the intrinsic pathway is mediated by stress-mediated damage such as alterations in temperature, osmolality, DNA damaging agents, free radical generation compounds, removal of nutrients, and oxygen deprivation (Duprez et al., 2009; Sprick & Walczak, 2004; Taylor et al., 2008; Wyllie, 2010). The activation of the two pathways leads to the same type of apoptotic response, causing release of cytochrome c (Cyt-C) from mitochondria and activation of the executioner caspase-3 (Duprez et al., 2009; Sprick & Walczak, 2004; Taylor et al., 2008). In the normal state, Akt activation (phospho-Akt, pAkt) by various growth factors induces cell survival via protection of mitochondrial integrity and inhibition of Cyt-C release (Gottlob et al., 2001; Plas et al., 2001; Uchiyama et al., 2004; Wyllie, 2010). Akt has also been reported to stimulate the expression of anti-apoptotic Bcl-2 proteins, such as Bcl-xL that prevents permeabilization of the mitochondrial membrane by inhibiting activation of Bas/Bak (Jones et al., 2000; Zong et al., 1999). However, under apoptotic stimulation, pro-apoptotic members of the Bcl-2 family (Bax, Bak, and Bad) are activated and induce the release of Cyt-C from mitochondria by binding and inactivating anti-apoptotic proteins (Gustafsson & Gottlieb, 2007; Scorrano & Korsmeyer, 2003). As the penultimate event, caspase-3 is activated to cleaved

caspase-3 to generate all the biochemical and morphologic hallmarks of cell apoptosis.

Platelets regulate the balance between apoptosis and cell survival in numerous cells involved in the repair of injured tissues (Freishtat et al., 2009; Gambim et al., 2007; Mause et al., 2010; Pakala, Willerson, & Benedict, 1994; Sharron et al., 2012; Stellos & Gawaz, 2007). Since platelets have a critical role in regulation of cell death and survival and contains various growth factors and cytokines (Fekete et al., 2012; Shih et al., 2011), we hypothesized that human platelet lysate (HPL) would increase ECFC survival and enhance ECFC vascularization by modulating the balance between apoptosis and cell survival of ECFC in 3D collagen matrices.

3.2. Materials And Methods

Culture of human umbilical cord blood derived ECFC

ECFC were isolated and cultured as previously described (Ingram et al., 2004). ECFC colonies appeared between 5 and 22 days of culture and were noted to form colonies of adherent cells with cobblestone morphology. After approximately 10 days of culture, the ECFC-derived ECs were released from the culture dish by TrypLE™ Express (Gibco) and replated onto 25/75 cm^2 tissue culture flasks pre-coated with Type I rat-tail collagen (BD Biosciences) for subsequent passage.

Preparation of three-dimensional (3D) collagen matrices

All of the type I collagen oligomers and associated polymerization reagents were purchased from GeniPhys (Bailey et al., 2011). Stock oligomer was diluted in 0.01N hydrochloric acid (HCl) and neutralized according to manufacturer's recommendations to achieve a final oligomer concentration of 1.37 mg/ml (200Pa matrix stiffness). ECFC (1 x 10^5 cells/60 µl or 1 x 10^6 cells/250 µl) were suspended in the collagen solution with or without human platelet lysate (HPL-10% of final concentration) at 4°C. The HPL was purchased from Gemeinnützige Salzburger Landeskliniken Betriebsges (SALK). The HPL was prepared from pooled platelet-rich plasma derived from a minimum of 40 whole blood donations (Schallmoser & Strunk, 2009). The collagen-cell suspensions were plated on 96-well or 48-well plates and allowed to polymerize at 37°C for 30 minutes and covered with complete endothelial cell growth medium (EGM-2, Lonza) with 10% defined fetal bovine serum (Hyclone) for incubation for one to three days at 37°C, 5% CO_2.

Toluidine blue staining of 3D collagen matrices and quantification of *in vitro* vascular structures

For analysis of *in vitro* vascular structure formation in 3D collagen matrix, cellularized collagen matrices were fixed with 4% paraformaldehyde for 20 minutes on Day 1 or Day 3 after implantation. The matrices were stained with 0.1% toluidine blue O dye (30% methanol) for 25 minutes at room temperature and washed with PBS for 30 minutes for three times at room temperature. Vascular

structures including vacuoles, lumens, and tube formation were quantified using ImageJ image analysis software (National Institutes of Health (NIH)). Vacuoles and lumens were defined as areas completely surrounded by a toluidine blue-labeled endothelial cell membrane. A single image of the entire well was captured at a depth of 10 microns from the surface of the matrix and then additional places 150 microns apart for each of triplicate samples of each group. Images of vascular structures were captured at 10x and 20x levels of magnification using a Leica DM IRE2 microscope (Leica Microsystems) with attached Retiga 4000R digital camera (QImaging).

Assessment of apoptosis of ECFC in 3D collagen matrices

Apoptosis was assessed by examining the percentage of human $CD31^+$ ECFC that bound Annexin V and propidium iodide and was performed as per the manufacturer's instruction (Apoptosis Detection kit, eBioscience). ECFC (1×10^5 cells/60 µl) were suspended in collagen matrices with/without HPL (10% of final concentration), with dimethyl sulfoxide (DMSO) vehicle (same volume of solvent for each inhibitor) or with signaling pathway inhibitors, plated in 96-well plates and incubated for one to three days as above. As previously described (H. Kim, Huang, et al., 2015), matrices were recovered from one to three days after implantation and incubated in 250 µl Collagenase Type I (0.25%) (Stemcell technology) for 20 minutes at 37°C. Akt1 inhibitor (A-674563) and Bcl-xL inhibitor (ABT-263/Navitoclax) were purchased from Selleckchem and Akt2 inhibitor (CCT128930) was purchased from Santa Cruz biotechnology. Cell dissociation

buffer was added to stop the enzymatic reaction (Invitrogen). Cells were centrifuged at 500 g for 5 minutes at room temperature. Cell pellets were suspended in staining buffer and stained with anti-human CD31 antibody conjugated to phycoerythrin (PE) for 15 minutes (clone WM-59, BD Biosciences Pharmingen). Cells were incubated and stained for Annexin V-allophycocyanin (APC) and propidium iodide (PI) in binding buffer for 10 minutes at room temperature in the dark. Stained cells were analyzed by FlowJo software. Three different clones of ECFCs were analyzed in each experiment and each experiment repeated one or two times.

Western blot

Cellular protein lysates were extracted from the ECFC collagen matrix implants and electrophoresed using sodium dodecyl sulfate–polyacrylamide matrix electrophoresis (SDS-PAGE), transferred to nitrocellulose, and probed with monoclonal antibodies that included anti-human Bcl-xL (clone 54H6, Cell signaling), cleaved caspase-3 (clone 3G2, Cell signaling), Akt1 (clone 2H10, Cell signaling), Akt2 (clone L79B2, Cell signaling), phospho-Akt1 (pAkt1 Ser473) (clone D7F10, Cell signaling), phospho-Akt2 (pAkt2 Ser474) (clone D3H2, Cell signaling), and phospho-Bad (pBad Ser136) (clone D25H8, Cell signaling). Anti-human β-actin (mAbcam 8226, Abcam) and GAPDH (clone 14C10, Cell signaling) antibodies were used to probe for loading control proteins. Three different clones of ECFCs were analyzed in each experiment on at least two occasions.

Implantation of human cord blood derived ECFC into NOD/SCID mice

Cellularized matrix implants were cast as noted above. Cultured ECFC (1 x 10^6 cells/250 µl) were suspended in the collagen mixture with or without HPL (10% of final concentration). Then 250 µL of the cell suspension was pipetted into one well of a 48-well tissue culture plate, allowed to polymerize at 37°C for 30 minutes, and covered with 500 µL EGM-2 medium for 30 minute incubation at 37°C, in 5% CO_2. Matrices were implanted into the flanks of anesthetized 6-9-week-old NOD/SCID mice. After 5 days or 14 days, the mice were sacrificed; the grafts were excised and analyzed by histology and immunohistochemistry (N=6 each group) as previously described (L. Huang et al., 2011; Yoder et al., 2007).

Histology and immunohistochemistry

Sections were stained as previously described (Yoder et al., 2007). Briefly, paraffin-embedded tissue sections were deparaffinized and then either directly stained with hematoxylin and eosin (H&E) or immersed in retrieval solution (Dako) for 20 minutes at 90–99°C. Slides were incubated at room temperature for 30 minutes with anti-human CD31 antibody (clone JC70/A, Abcam) followed by a 10-minute incubation with LASB2 link-biotin and streptavidin-HRP (Vector Laboratories), then developed with DAB (Vector Laboratories) solution for 5 minutes. Slides were analyzed by microscope under 20x magnification using a Leica DM 4000B microscope (Leica Microsystems) with attached Spot-KE digital camera (Diagnostic Instruments).

Statistical analysis

Results are expressed as mean ± the standard error of the mean (SEM) for the study variables. The assessment of apoptosis of ECFC, *in vitro* vascular structure quantification and the quantification of western blot data points (ECFC versus ECFC+HPL), western blot data points (ECFC treated with DMSO, Akt1 inhibitor, Akt2 inhibitor, or Bcl-xL inhibitor) were assessed by unpaired t-test. A statistically significant difference was set at *P<0.05, **P<0.001, or ***P<0.0001.

3.3. Results

Human cord blood (CB) derived ECFC with human platelet lysate (HPL) significantly reduces apoptosis of ECFC in three dimensional (3D) collagen matrices.

Our previous study demonstrated that implanted ECFC started undergoing apoptosis on day 1 and a significant majority of ECFC died on day 2 and 3 in 3D collagen matrices after implantation (H. Kim, Huang, et al., 2015). We tested whether the co-implantation of ECFC with HPL would decrease apoptosis of human CB ECFC in the 3D collagen implants *in vitro*. After 1-3 days, ECFC were recovered from 3D collagen matrices and assessed for apoptosis of ECFC stained with anti-human CD31, Annexin V and propidium iodide (PI). $CD31^{+}$ ECFC were selected from the total cell population and separated into Annexin $V^{+/-}$ and $PI^{+/-}$ expressing subsets (Figure 3.1.). Implanted ECFC without HPL started undergoing apoptosis on day 1 and a significant amount of the cells died by day 3 after matrix formation *in vitro* (Figure 3.2. a). In contrast, ECFC co-

implants with HPL revealed a significantly higher percentage of the Annexin V^- /PI^- viable cell population compared to the control (ECFC alone) (Figure 3.2. a). We investigated whether co-implantation of ECFC with HPL would enhance more vascular structures in 3D collagen matrices *in vitro*. ECFC-derived vascular structures were defined as endothelial cells with vacuoles and lumenized structures were defined as areas completely surrounded by a toluidine blue-labeled endothelial cell membrane (Figure 3.2. b). A significantly greater average number of vascular structures (Figure 3.2. c) and total luminal area of vascular structures (mm^2) (Figure 3.2. d) were quantified in ECFC+HPL implants compared to the control implants on both day 1 and 3 of the *in vitro* study. These data suggest that ECFC+HPL co-implantation significantly promotes vasculogenesis by inducing greater cell survival of ECFC in 3D collagen matrices *in vitro*.

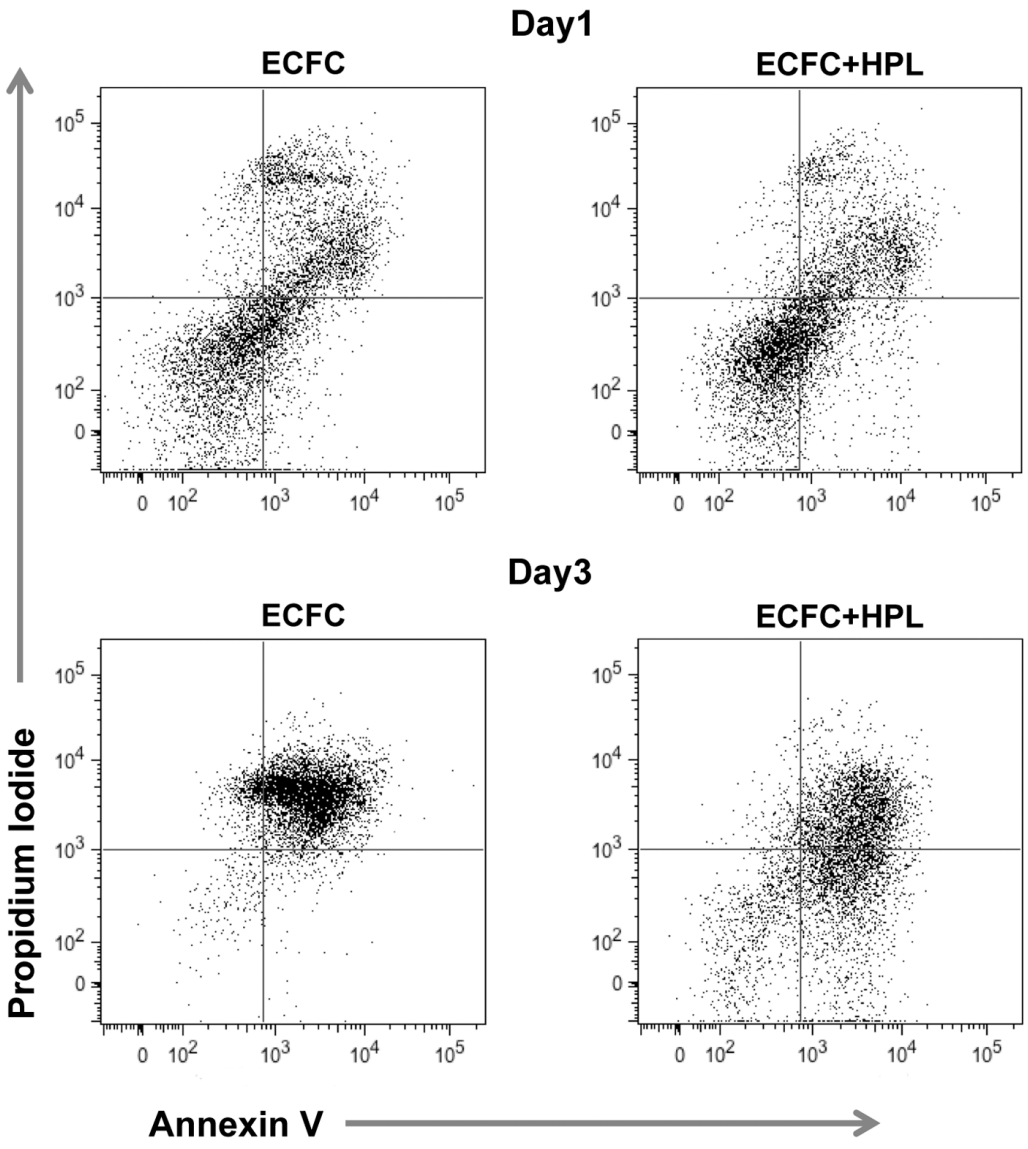

Day1
ECFC
ECFC+HPL
Day3
ECFC
ECFC+HPL
Propidium Iodide
Annexin V
0
10^2
10^3
10^4
10^5

Figure 3.1. Co-implantation of ECFC with HPL promotes cell survival of ECFC in 3D collagen matrices. Representative dot plots of apoptosis analysis of co-implant of ECFC alone and ECFC with HPL on day 1 and 3 of *in vitro* culture. Reprinted with permission from “Human platelet lysate improves human cord blood derived ECFC survival and vasculogenesis in three dimensional (3D) collagen matrices” by Kim et al., 2015, Microvascular Research, 101:72-81. Figure 3.1. was from my original work (H. Kim, Prasain, et al., 2015).

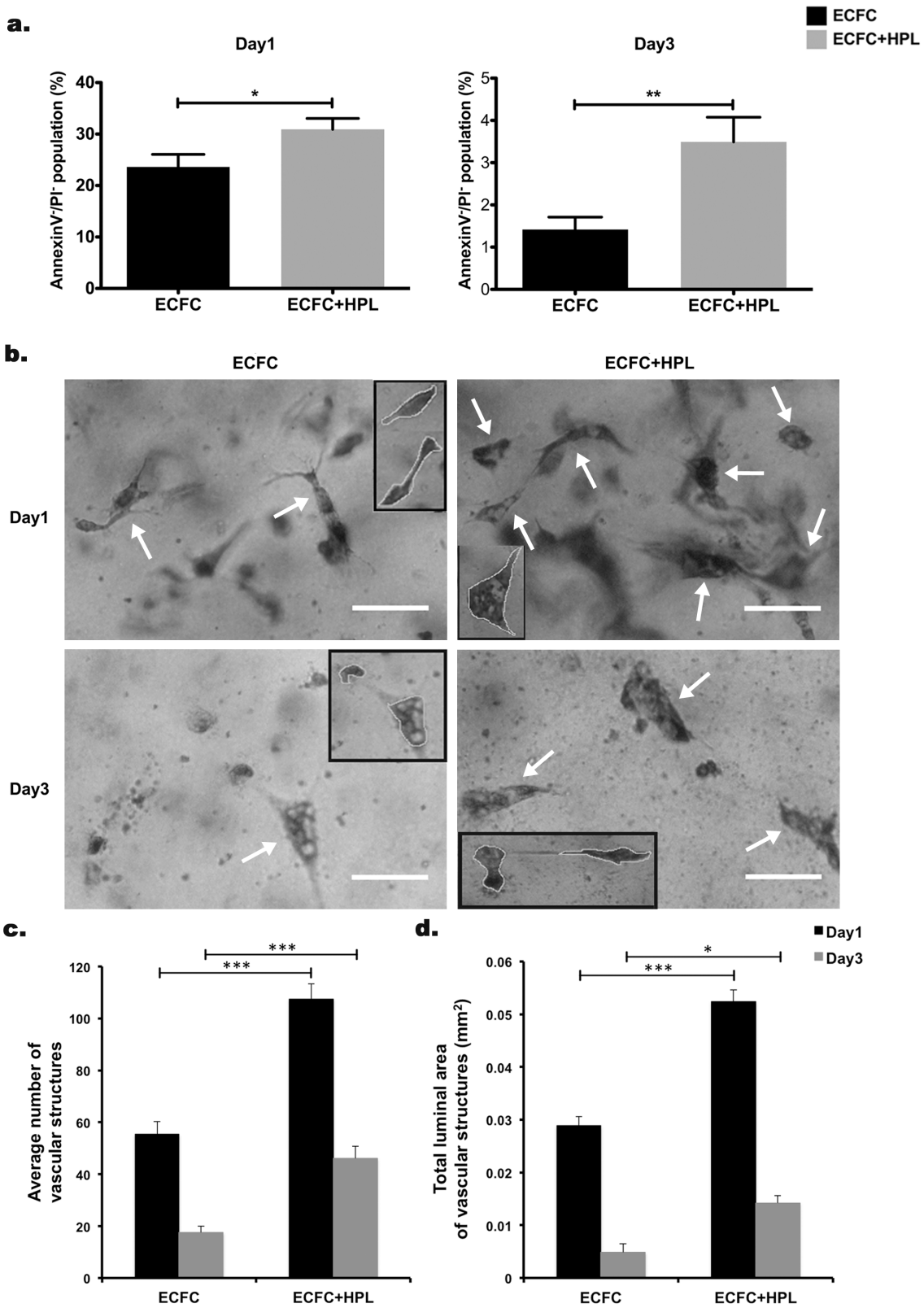

a.
ECFC
ECFC+HPL
Day1
Day3
AnnexinV-/PI- population (%)
*
**
ECFC
ECFC+HPL
b.
ECFC
ECFC+HPL
Day1
Day3
c.
Average number of vascular structures
***
***
d.
Day1
Day3
Total luminal area of vascular structures (mm2)
*
***

Figure 3.2. Co-implantation of ECFC with HPL diminishes apoptosis of ECFC in 3D collagen matrices *in vitro*. (a) The Annexin V^-/PI^- viable population of co-implanted ECFC with HPL was significantly higher on day 1 and 3 compared to implantation of ECFC alone. N=3. *P<0.05. (b) Representative images of vascular structures of ECFC implanted with or without HPL in collagen matrices on day 1 and 3 (white arrows indicate the structures and small boxes indicates representative counted structures). Scale bar represents 50 μm. Co-implantation of ECFC with HPL was resulted in a significant increase in (c) the average number of vascular structures per collagen matrix surface area and (d) total luminal area of vascular structures per matrix surface area (mm^2) compared to controls. N=10 each group. *P<0.05, **P<0.001, or ***P<0.0001. Reprinted with permission from "Human platelet lysate improves human cord blood derived ECFC survival and vasculogenesis in three dimensional (3D) collagen matrices" by Kim et al., 2015, Microvascular Research, 101:72-81. Figure 3.2. was from my original work (H. Kim, Prasain, et al., 2015).

Co-implantation of ECFC with HPL modulates the balance between apoptosis and cell survival in 3D collagen matrices.

Knowing that HPL treatment can modulate the balance between apoptosis and cell survival (Freishtat et al., 2009; Gambim et al., 2007; Mause et al., 2010; Pakala et al., 1994; Sharron et al., 2012; Stellos & Gawaz, 2007), we subsequently tested whether ECFC implantation with HPL would alter the protein expression level of pro-apoptotic, anti-apoptotic, and pro-survival molecules *in vitro*. On day 1 and 3 after casting the matrices, expression of pro-survival molecules (Akt1 and phosphorylated Akt1 (pAkt1)) and an anti-apoptotic molecule (Bcl-xL) were upregulated in implants of ECFC with HPL compared to ECFC implants without HPL (Figure 3.3. a and b). The pro-apoptotic molecule, Bad was phosphorylated (pBad) to an inactive form that blocks apoptosis and the expression level of pBad was upregulated in ECFC implants with HPL compared to control implants on day 1 and 3 *in vitro* (Figure 3.3. a and b). The expression level of active cleaved caspase-3 was enhanced in co-implants of ECFC with HPL compared to control samples on day 1 and 3 (Figure 3.3. a and b). We also examined protein expression level of Akt2 and pAkt2, but these proteins were not detected in either group on day 1 or 3 (date not shown). Since HPL contains various cytokines and growth factors that are involved in pro-survival and anti-apoptotic signaling (Bendinelli et al., 2010; El-Sharkawy et al., 2007; Fekete et al., 2012), HPL may induce more pro-survival/anti-apoptotic signaling to resist the apoptotic stimuli that stimulate activation of caspasae-3 (cleaved caspase-3). These data suggest that co-implantation of ECFC with HPL *in vitro* promotes cell

survival of ECFC by modulating the balance between pro-apoptotic and anti-apoptotic/pro-survival molecules.

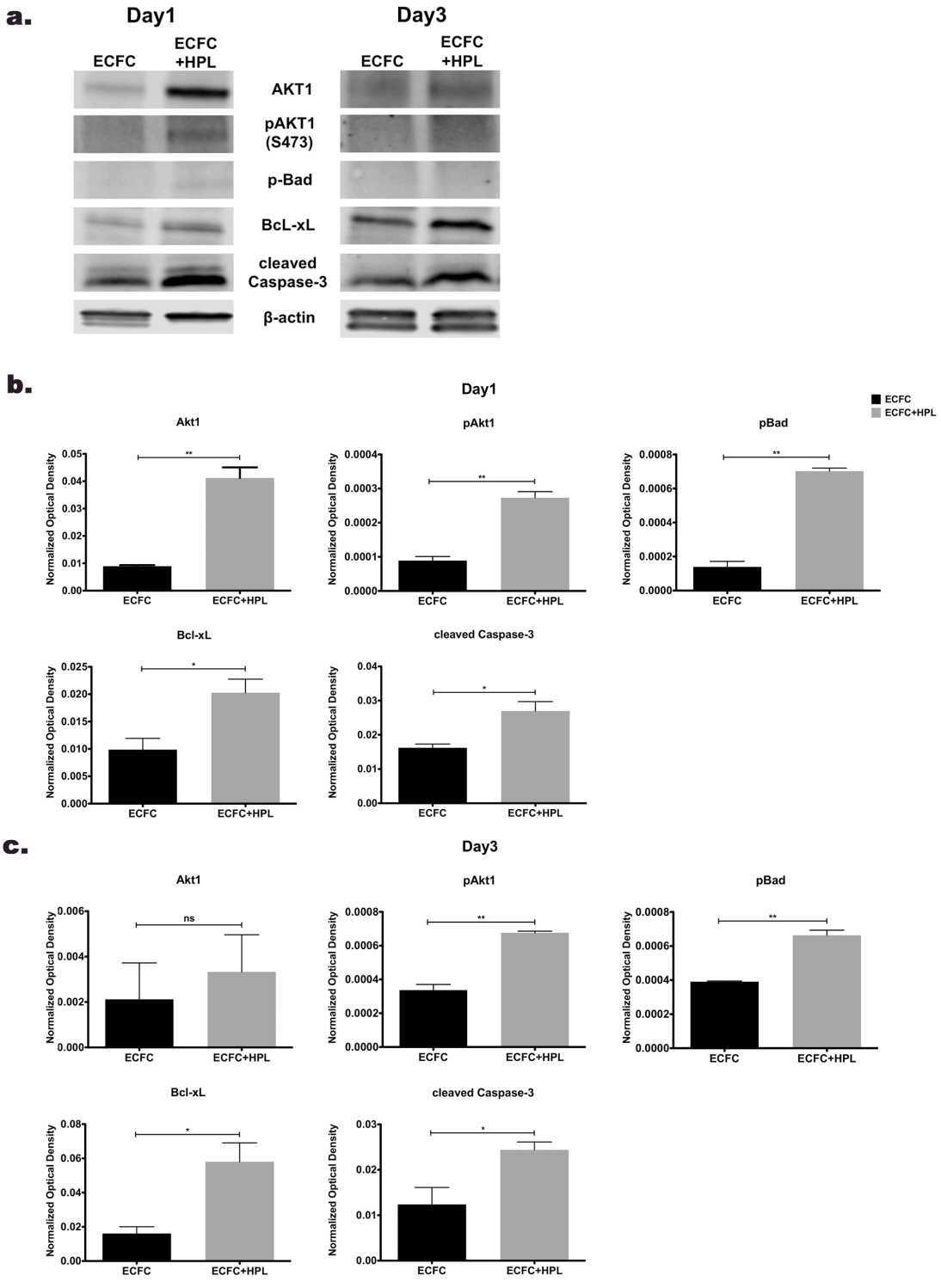

a.
Day1
Day3
ECFC
ECFC +HPL
ECFC
ECFC +HPL
AKT1
pAKT1 (S473)
p-Bad
BcL-xL
cleaved Caspase-3
β-actin
b.
Day1
ECFC
ECFC+HPL
Akt1
pAkt1
pBad
Bcl-xL
cleaved Caspase-3
Normalized Optical Density
ECFC
ECFC+HPL
c.
Day3
Akt1
pAkt1
pBad
Bcl-xL
cleaved Caspase-3
ns
*
**

Figure 3.3. HPL in ECFC implant stimulates protein expression of apoptotic/cell survival related molecules. (a) Representative immunoblots of pro-survival (Akt1, pAkt1, pBad, and Bcl-xL) and apoptotic (cleaved caspase-3) proteins from implants of ECFC and ECFC with HPL. Lower panels show quantification of repeated experiments on day 1 (b) and day 3(c) after implantation (protein expression levels were normalized using β-actin). Pro-survival molecules and apoptotic molecule were significantly upregulated on co-implantation of ECFC with HPL compared to ECFC alone on day 1 and day 3. N=2 each group. ns=no significance. *P<0.05 or **P<0.001. Reprinted with permission from “Human platelet lysate improves human cord blood derived ECFC survival and vasculogenesis in three dimensional (3D) collagen matrices” by Kim et al., 2015, Microvascular Research, 101:72-81. Figure 3.3. was from my original work (H. Kim, Prasain, et al., 2015).

Co-implantation of ECFC with HPL significantly enhances vasculogenesis of ECFC *in vivo*.

ECFC implantation with HPL induced more formation of vascular structures *in vitro* in 3D collagen matrices compared to control matrices (Figure 3.2.). We subsequently tested whether co-implantation of ECFC with HPL would promote vasculogenesis in an *in vivo* model. Specific anti-human CD31 antibody staining revealed human CB ECFC cell-lined microvessels that were perfused with murine red blood cells in the grafts (Figure 3.4. a). The quantification of murine erythrocyte-containing human $CD31^+$ microvessels revealed a significant increase of average vessel numbers (Figure 3.4. b) and total vessel area (mm^2) (Figure 3.4. c) in ECFC+HPL implants compared to ECFC alone implants. In addition, the size distribution of the microvessels differed between ECFC implants with HPL and ECFC alone implants (Figure 3.4. d). Noticeably, only co-implantation of ECFC with HPL gave rise to the largest vascular elements (1001-4000 μm^2). These data suggest that co-implantation of ECFC with HPL enhanced vasculogenesis *in vivo* as evidenced by more ECFC-derived vessel formation with an enlarged vascular bed area.

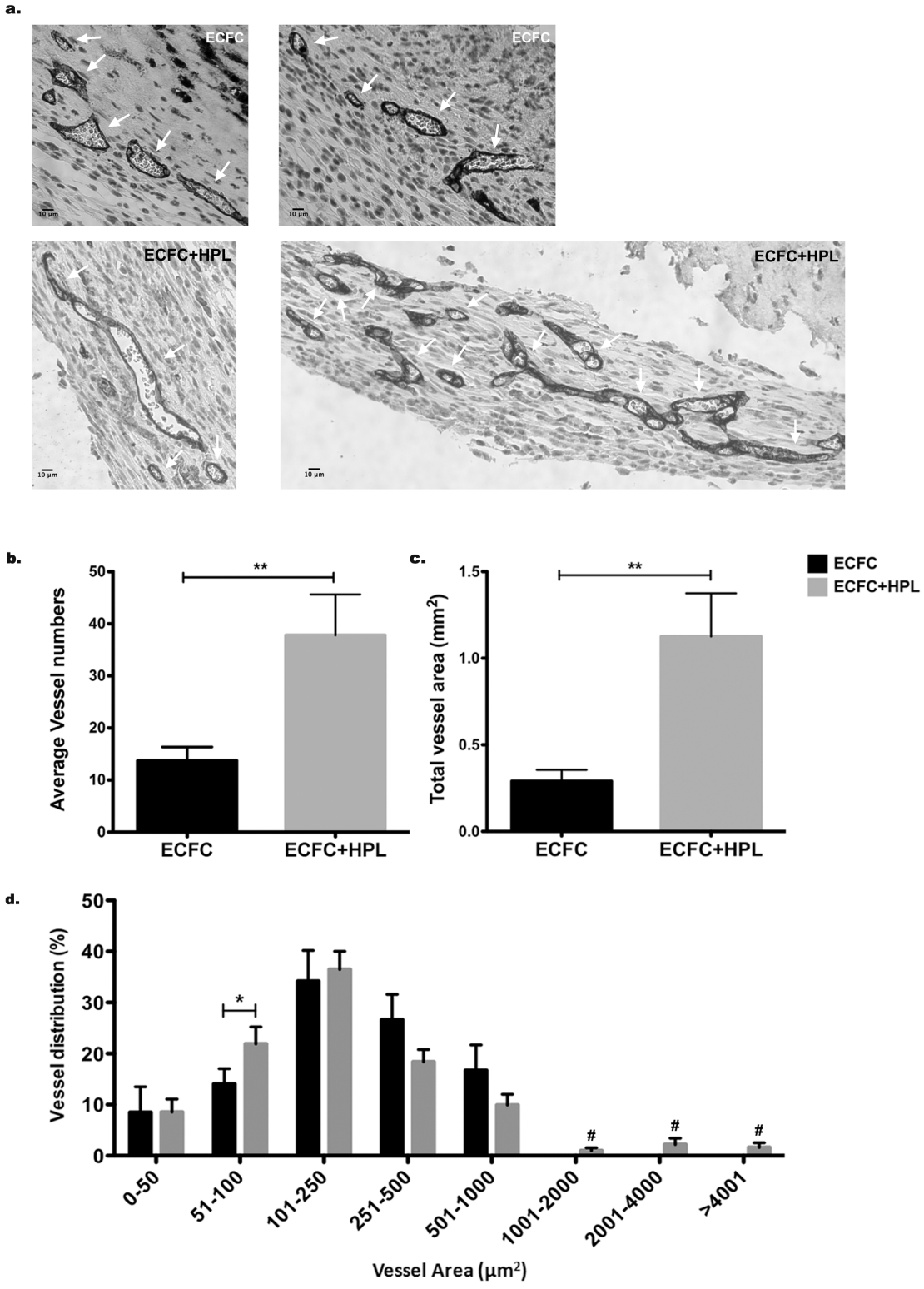
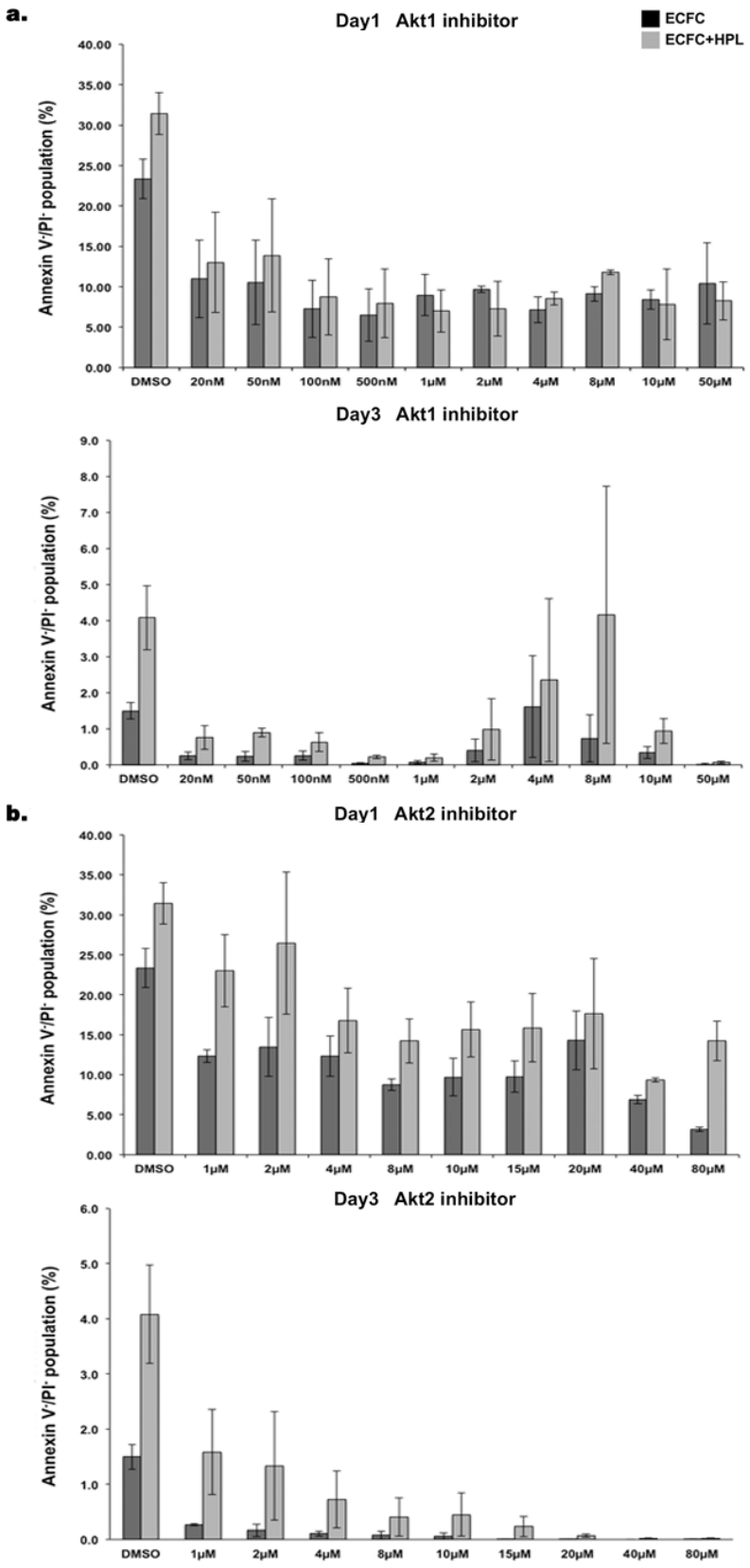

a.
ECFC
ECFC
ECFC+HPL
ECFC+HPL
10 µm
b.
Average Vessel numbers
**
ECFC
ECFC+HPL
c.
Total vessel area (mm²)
**
ECFC
ECFC+HPL
d.
Vessel distribution (%)
*
#
0-50
51-100
101-250
251-500
501-1000
1001-2000
2001-4000
>4001
Vessel Area (µm²)

Figure 3.4. Co-implantation of ECFC with HPL induces the formation of functional vessels *in vivo*. (a) Human ECFC-derived vessels were identified by rat anti-human CD31 staining (white arrows indicate ECFC-derived $CD31^+$ vessels). The two top panel images show representative images of ECFC-derived human $CD31^+$ vessels. The two lower panel images show representative images of ECFC with HPL derived human $CD31^+$ vessels. Scale bar represents 10μm. There was a significant increase in (b) average vessel numbers per unit sectional area and (c) total vessel area (mm^2) per unit sectional area in the ECFC with HPL co-implants. (d) In addition, only co-implants of ECFC with HPL formed the largest sized vessels (# indicates 1001-4000 μm^2) in the size distribution of $hCD31^+$ microvessels perfused with murine red blood cells. N=10 mice each group. *P<0.05 or **P<0.001. Reprinted with permission from "Human platelet lysate improves human cord blood derived ECFC survival and vasculogenesis in three dimensional (3D) collagen matrices" by Kim et al., 2015, Microvascular Research, 101:72-81. Figure 3.4. was from my original work (H. Kim, Prasain, et al., 2015).

Inhibition of pro-survival/anti-apoptotic signaling enhances apoptosis of ECFC by modulating protein expression of pro-survival molecules in 3D collagen matrices

Since ECFC cell survival was significantly increased when ECFC were co-implanted with HPL by balancing the anti-apoptotic/pro-survival (Akt1, pAkt1, pBad, and Bcl-xL) and pro-apoptotic signals (cleaved caspase-3), we evaluated whether inhibition of these pro-survival and anti-apoptotic molecules would alter cell survival of ECFC in 3D collagen matrices. Akt is a critical regulator of PI3K-mediated cell survival (Franke et al., 1995; Yao & Cooper, 1995). Since, Akt1 and Akt2 are the upstream molecules in PI3K-mediated cell survival and have been reported to display compensatory functions between isoforms (W. S. Chen et al., 2001; Paradis & Ruvkun, 1998), Akt1 and Akt2 inhibitors were selected to examine the importance of these signaling pathways on ECFC pro-survival signaling. Akt1 inhibitor blocks the phosphorylation of the Akt downstream target pBad to result in increased apoptosis (Luo et al., 2005; Zhu et al., 2008). Akt2 inhibitor has been reported as a novel small molecule that inhibits cell proliferation by inducing cell cycle arrest and thereby triggering apoptosis (Yap et al., 2011). Also, the inhibitor of Bcl-xL was selected for blocking anti-apoptotic signaling in ECFC implants. Bcl-xL inhibitor binds to apoptosis suppressor Bcl-xL and prevents binding to the apoptotic effectors Bax and Bak proteins (Shoemaker et al., 2008; Tse et al., 2008). The concentration of each inhibitor was chosen based on dose-dependent optimization experiments (Figure. 3.5.). ECFC and ECFC+HPL implants were treated with DMSO as vehicle control, 2

μm of Akt1 inhibitor (Figure 3.6. a), 2 μm of Akt2 inhibitor (Figure 3.6. b), and 20 nM of Bcl-xL inhibitor (Figure 3.6. c). The percentage of the Annexin V^-/PI^- viable population was significantly reduced in both implants of ECFC alone and ECFC with HPL when exposed to the Akt1 inhibitor on both day 1 and 3 of culture. While cell survival of ECFC alone implants were significantly decreased on day 1, both implants of ECFC and ECFC+HPL displayed zero cell survival on day 3 when treated with the Bcl-xL inhibitor. Akt2 inhibitor treatment significantly reduced cell survival of ECFC alone in implants on day 1 and 3. However, there was not a statistical significant difference in cell survival of ECFC in the ECFC+HPL implants when treated with Akt2 inhibitor on day 1 and 3 because of high variability among the repeated experiments. As a result, only Akt1 inhibition of ECFC displayed a significant reduction of ECFC cell survival in both implants on day 1 following implantation. These results implicate the Akt1 pathway as the most important pro-survival pathway promoting ECFC survival in the 3D matrices.


a.
Day1 Akt1 inhibitor
ECFC
ECFC+HPL
Annexin V+/PI- population (%)
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
DMSO
20nM
50nM
100nM
500nM
1μM
2μM
4μM
8μM
10μM
50μM
Day3 Akt1 inhibitor
Annexin V+/PI- population (%)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
DMSO
20nM
50nM
100nM
500nM
1μM
2μM
4μM
8μM
10μM
50μM
b.
Day1 Akt2 inhibitor
Annexin V+/PI- population (%)
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
DMSO
1μM
2μM
4μM
8μM
10μM
15μM
20μM
40μM
80μM
Day3 Akt2 inhibitor
Annexin V+/PI- population (%)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
DMSO
1μM
2μM
4μM
8μM
10μM
15μM
20μM
40μM
80μM

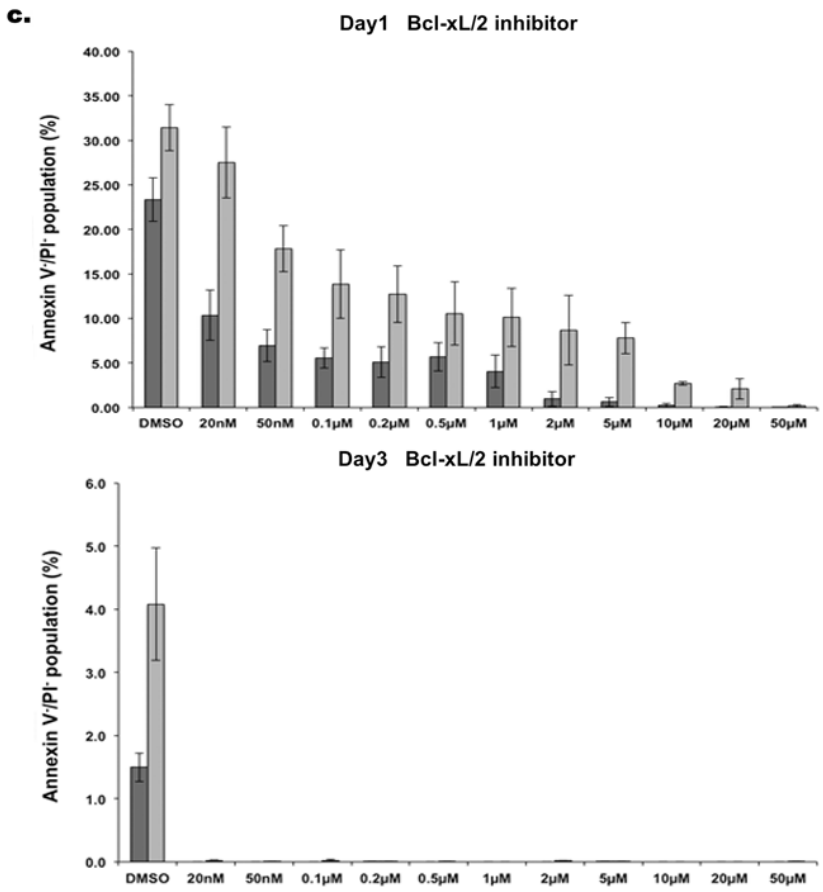


Figure 3.5. The effect of inhibitors of Akt1, Akt2, and Bcl-xL on ECFC in 3D collagen matrices on day1 and day3. Percentage of the Annexin V^-/PI^- viable population indicates the dose-dependent inhibition of cell survival in ECFC via treatment of DMSO, (a) Akt1 inhibitor, (b) Akt2 inhibitor, or (c) Bcl-xL inhibitor. Reprinted with permission from "Human platelet lysate improves human cord blood derived ECFC survival and vasculogenesis in three dimensional (3D) collagen matrices" by Kim et al., 2015, Microvascular Research, 101:72-81. Figure 3.5. was from my original work (H. Kim, Prasain, et al., 2015).

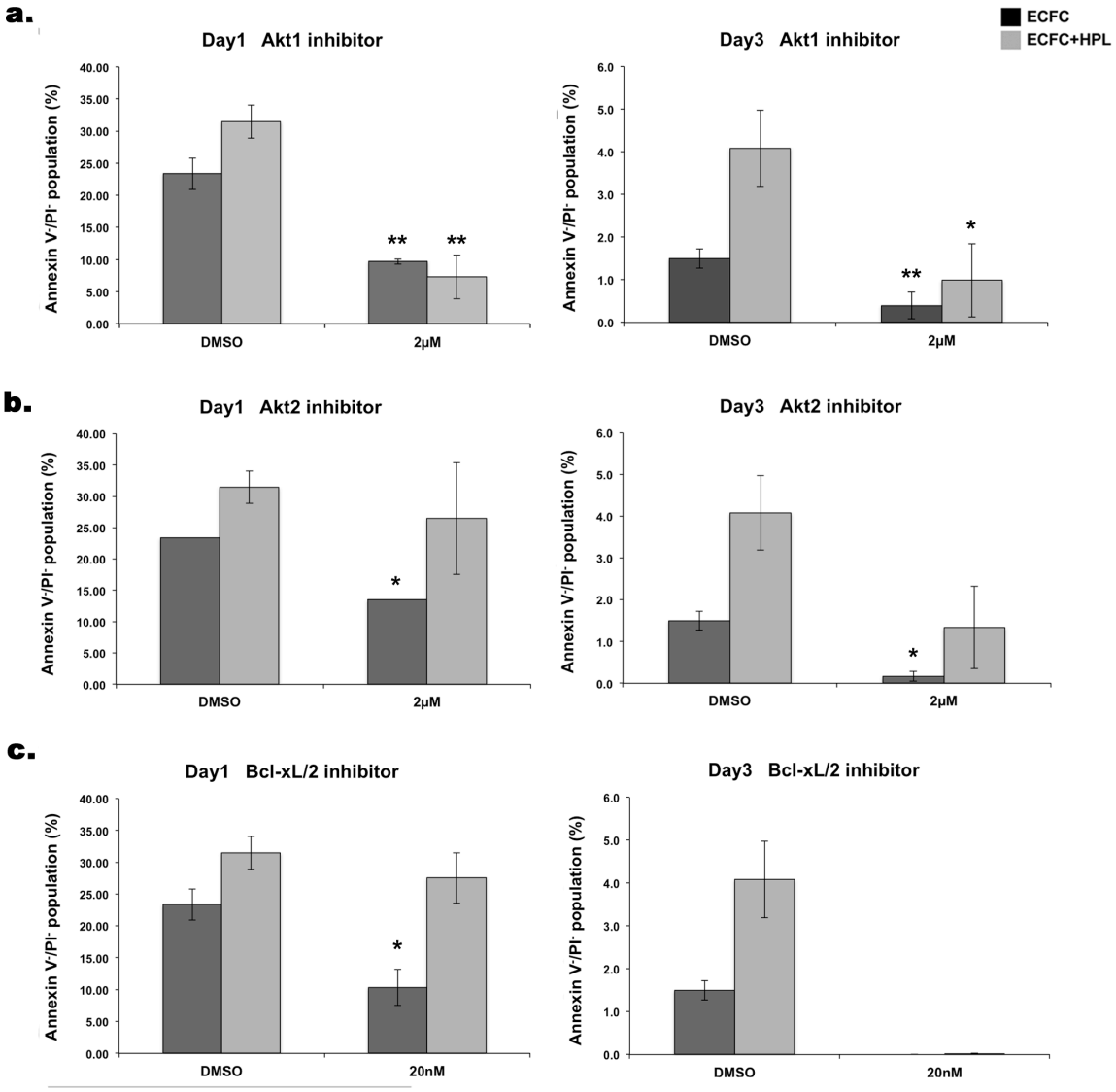

a.
ECFC
ECFC+HPL
Day1 Akt1 inhibitor
Day3 Akt1 inhibitor
Annexin V-/PI- population (%)
DMSO
2μM
b.
Day1 Akt2 inhibitor
Day3 Akt2 inhibitor
c.
Day1 Bcl-xL/2 inhibitor
Day3 Bcl-xL/2 inhibitor
20nM

Figure 3.6. Inhibition of pro-survival molecules reduces cell survival in 3D collagen matrices on day 1 and day 3. The Annexin V^-/PI^- viable population of inhibitor treatment of ECFC alone or ECFC with HPL in 3D collagen matrices on day 1 and day 3: DMSO as vehicle control, (a) Akt1 inhibitor, 2 μm, (b) Akt2 inhibitor, 2 μm, and (c) Bcl-xL inhibitor, 20 nM. Percentage of Annexin V^-/PI^- viable population was significantly decreased by inhibition of Akt1, Akt2, and Bcl-xL. Of note, the most profound decrease in viability was observed in the ECFC+HPL matrices that contained the Akt1 inhibitor on day 1. N=3 each group. *P<0.05 or **P<0.001. Reprinted with permission from "Human platelet lysate improves human cord blood derived ECFC survival and vasculogenesis in three dimensional (3D) collagen matrices" by Kim et al., 2015, Microvascular Research, 101:72-81. Figure 3.6. was from my original work (H. Kim, Prasain, et al., 2015).

Next, we examined whether the inhibitor treatments would alter the protein expression level of pro-survival/anti-apoptotic or pro-apoptotic molecules in ECFC in 3D collagen matrices on day 1. We tested only day 1 implants because cell survival of ECFC was close to zero by all inhibitor treatments on day 3. When treated with the Akt1 inhibitor, pBad (inactive form that blocks apoptosis) was barely detected in ECFC alone implants and significantly decreased in ECFC+HPL implants compared to DMSO control groups. The level of Bcl-xL (anti-apoptotic molecule) was not changed in both implantation of ECFC and ECFC+HPL matrices compared to DMSO control samples. When exposed to the Bcl-xL inhibitor, the level of pBad and Bcl-xL was significantly decreased in the both groups compared to DMSO controls. On the other hand, Akt2 inhibition caused a significant increase of expression level of Akt1/pAkt1, Bcl-xL (anti-apoptotic molecule), and pBad (inactivated pro-apoptotic molecule) in both groups compared to DMSO groups (Figure 3.7.). Cleaved caspase-3 (pro-apoptotic molecule) was increased with treatment of the three inhibitors in implants of ECFC alone compared to ECFC implants with DMSO (Figure 3.7. b). However, cleaved caspase-3 expression was not changed in ECFC+HPL implants with the three inhibitors compared to ECFC+HPL implants treated with DMSO samples (Figure 3.7. c). Thus, inhibition of Akt1 and Bcl-xL (pro-survival/anti-apoptotic molecules) promotes apoptosis of ECFC by decreasing pBad (inactivated pro-apoptotic molecule) and Bcl-xL (pro-survival molecule), which are Akt downstream substrates, in both ECFC alone and ECFC+HPL 3D implants. In summary, only Akt1 inhibition significantly diminished cell survival of

ECFC by decreasing protein level of pBad in ECFC implants with HPL, even though cleaved caspase-3 expression was not concomitantly changed.

In addition, we also determined that ECFC treated with the Akt1, Akt2, and Bcl-xL inhibitors on day 1 and 3 displayed significantly diminished vascular structures on day 1 and absence of vascular structures on day 3 (date not shown). Collectively, these observations have demonstrated that inhibition of the Akt1 signaling pathway significantly diminished cell survival and vasculogenesis of ECFC by altering expression level of pro-apoptotic and anti-apoptotic/pro-survival molecules.

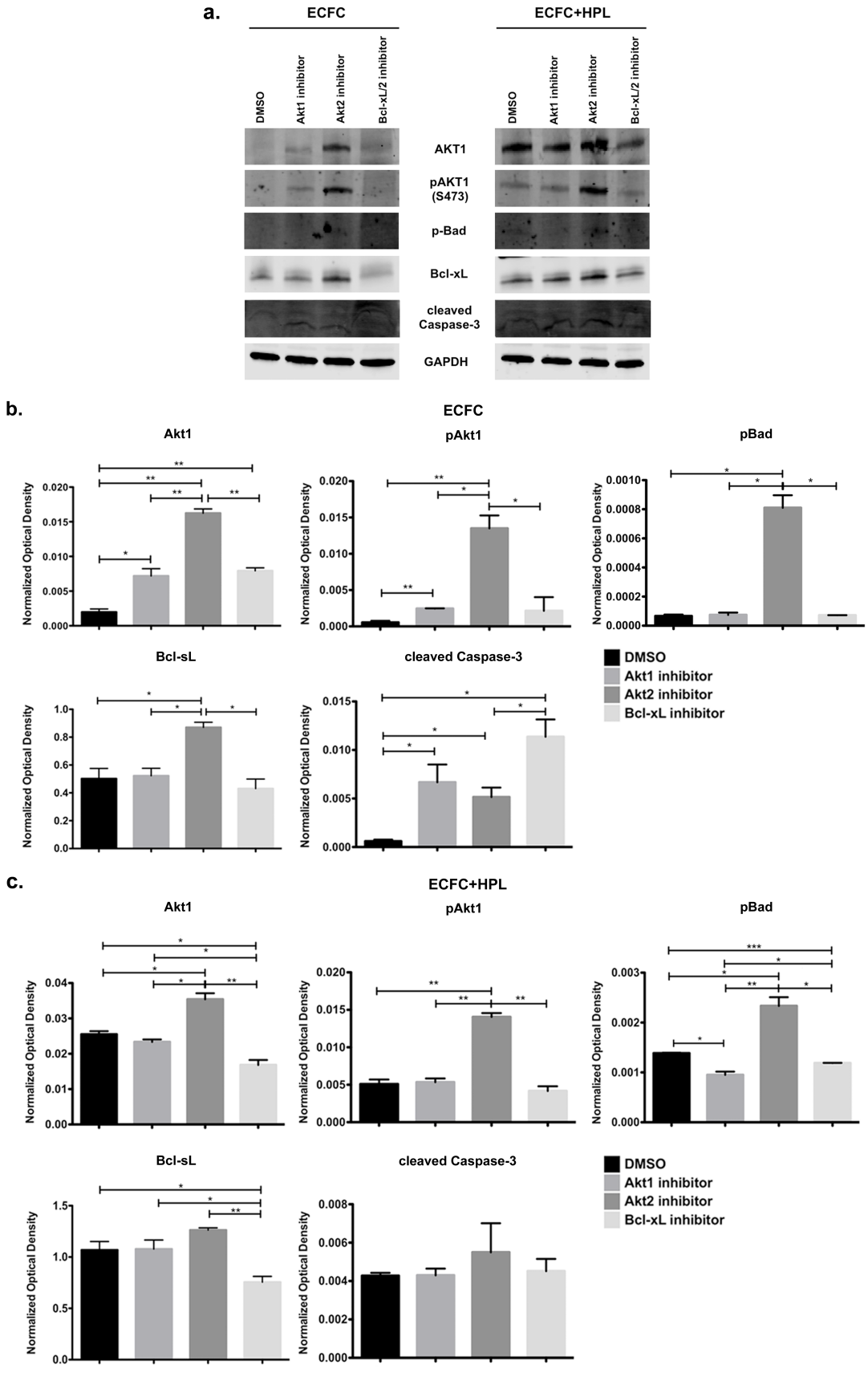

a.
ECFC
ECFC+HPL
DMSO
Akt1 inhibitor
Akt2 inhibitor
Bcl-xL/2 inhibitor
AKT1
pAKT1 (S473)
p-Bad
Bcl-xL
cleaved Caspase-3
GAPDH
b.
ECFC
Akt1
pAkt1
pBad
Bcl-sL
cleaved Caspase-3
Normalized Optical Density
DMSO
Akt1 inhibitor
Akt2 inhibitor
Bcl-xL inhibitor
c.
ECFC+HPL
Akt1
pAkt1
pBad
Bcl-sL
cleaved Caspase-3
Normalized Optical Density
DMSO
Akt1 inhibitor
Akt2 inhibitor
Bcl-xL inhibitor

Figure 3.7. Inhibition of pro-survival molecules affects protein expression of molecules involved in the apoptotic/cell survival signaling in 3D collagen matrices. (a) Representative immunoblots of inhibitor treatment of ECFC or ECFC with HPL in 3D collagen matrices (DMSO as vehicle control, Akt1 inhibitor, 2 μm, Akt2 inhibitor 2 μm, and Bcl-xL inhibitor 20 nM). Lower panels show quantification of repeated experiments of the inhibitor treatment ECFC alone (b) and ECFC with HPL (c) in 3D collagen matrix on day 1 after implantation (protein expression levels were normalized using GAPDH). Y axis=Normalized optical density N=2 each group. *P<0.05, **P<0.001, or ***P<0.0001. Reprinted with permission from “Human platelet lysate improves human cord blood derived ECFC survival and vasculogenesis in three dimensional (3D) collagen matrices” by Kim et al., 2015, Microvascular Research, 101:72-81. Figure 3.7. was from my original work (H. Kim, Prasain, et al., 2015).

3.4. Discussion

The microvascular network has dynamic structural plasticity. Vasculogenesis, angiogenesis, and microvascular remodeling are complex processes following endothelial cell growth, migration, and differentiation (Adams & Alitalo, 2007; Bader, Rayburn, Crowley, & Hynes, 1998; Carmeliet, 2000; Carmeliet et al., 1996; Coultas et al., 2005; Ferrara, 1999; Ferrara et al., 1996; Hanahan & Folkman, 1996; Lamalice et al., 2007; Risau, 1995, 1997; Shalaby et al., 1997). Many of theses *in vivo* processes can be modeled and examined *in vitro*. Human CB ECFC or human umbilical vein endothelial cells (HUVEC) participate in cell-cell and/or cell-matrix interactions and form capillary network within the first 24hrs on 2D Matrigel coated dishes. The capillary network formation shows tremendous structural plasticity and dynamic processes of not only new network formation but also network regression. By blocking cell-matrix interactions with colchicine or anti-human avb3 antibody, HUVEC failed to form a capillary network, and a significant increase of endothelial cell death was observed (Pollman et al., 1999). Thus, cell-matrix interactions are necessary for cell viability and the formation of a capillary network on Matrigel coated dishes (Pollman et al., 1999; Shih et al., 2011). Bcl-2 over expression in HUVEC enhanced cell viability and promoted stabilization of the capillary network formed on Matrigel (Pollman et al., 1999). This study suggested that the formation of capillary networks is dependent on the balance between pro-apoptotic and pro-survival of endothelial cells on 2D Matrigel substrates. We suggest that the balance between pro-apoptotic and anti-apoptotic/pro-survival signals play a critical role in promoting cell survival of

ECFC and enhancing vasculogenesis when ECFC are co-implanted with HPL in *in vitro and in vivo* models (Figure 3.2., 3.3., and 3.4.).

Previous studies have been reported that HPL contains various cytokines and growth factors that are involved in various cellular events, particularly cellular migration, proliferation, differentiation, extracellular matrix organization and remodeling, and cell survival (Aarabi et al., 2007; Fekete et al., 2012; Shih et al., 2011; Singer & Clark, 1999). Growth factors in HPL, especially, VEGF and angiopoietin-1, stimulate Akt to promote cell survival and ensure adequate vascular development in cardiovascular functions (Carmeliet et al., 1999; Fujio & Walsh, 1999; Gerber et al., 1998; I. Kim et al., 2000; Kontos et al., 1998; Papapetropoulos et al., 2000). Akt also has been reported to stimulate the expression of anti-apoptotic Bcl-2 proteins, such as Bcl-xL through the activation of NF-κB (I. Kim et al., 2000; Zong et al., 1999). Constitutive activation of Akt signaling protects cardiomyocytes against apoptosis (Fujio et al., 2000). In addition, chronic activation of Akt/PI3K pathway in endothelial cells enhances cell survival of endothelial cells (ECs) and promotes more vasculogenesis of ECs in absence of growth factors and serum (Seandel et al., 2008). Since Akt1 is generally the predominant expressed isoform (Akt1, Akt2, and Akt3) and compensates Akt2 functions for the inhibition of Akt2 (Ak2 knockout) (W. S. Chen et al., 2001; Paradis & Ruvkun, 1998), such compensation may explain the why inhibition of Akt2 may not effectively alter expression of pro-survival/anti-apoptotic survival signaling molecules in the ECFC in 3D matrices (Figure 3.6.

and 3.7.). Only Akt1 inhibition significantly decreased the downstream targets, pBad and significantly diminished cell survival of ECFC on both day 1 and day 3 in our *in vitro* model. Thus, growth factors in HPL may enhance cell survival of ECFC by activating Akt1 and enhancing expression of pro-survival/anti-apoptotic molecules (pAkt1, pBad, and Bcl-xL) against the apoptotic signal (cleaved caspase-3) mediated by environmental stress in our experimental models.

In summary, we have demonstrated that co-implantation of human cord blood ECFCs with HPL improves cell survival of ECFC and promotes robust formation of human ECFC-derived microvessels with increased average vessel number, total vessel area, and enlarged vessel sizes *in vivo*. By activating Akt1 pro-survival signaling in the ECFC, HPL modifies the balance of pro-apoptotic and anti-apoptotic/cell survival signaling in the implanted cells to enhance vasculogenesis.

CHAPTER 4. INHIBITION OF APOPTOSIS OF ECFC ENHANCES HUMAN CORD BLOOD DERIVED ECFC SURVIVAL AND VASCULOGENESIS IN THREE DIMENSIONAL (3D) MATRICES

4.1 Introduction

Endothelial progenitor cells (EPCs) play critical roles in the formation of new vessels or the recovery of damaged vascular endothelium through angiogenesis and vasculogenesis (Asahara et al., 1997; Rafii et al., 2002; Shi et al., 1998). Endothelial colony forming cells (ECFC) are derived from a rare circulating subset of EPCs that may arise from resident endothelium of established blood vessels in man (Hirschi et al., 2008). We have successfully isolated ECFC from human peripheral blood (PB) or umbilical cord blood (CB) and identified a hierarchy of proliferative potential in ECFC through the use of single cell clonogenic and functional assays (L. Huang et al., 2011; Ingram et al., 2004). Human CB derived ECFC also display *de novo* vessel forming ability in immunodeficient mice *in vivo* after subcutaneous implantation of ECFC in collagen-fibronectin matrices and upon inosculation with host murine vessels, become a part of the systemic host circulation (Au, Daheron, et al., 2008; Cheng et al., 2011; Critser et al., 2010; L. Huang et al., 2011; Melero-Martin et al., 2008; Melero-Martin et al., 2007; Reinisch et al., 2009; Whittington et al., 2013; Yoder et al., 2007).

In subcutaneous implants, human ECFC-derived vessels were detected as early as day 1 or 2 after implantation in non-obese diabetic/severe combined immunodeficient mice (NOD/SCID) but these nascent vessels were not properly perfused with the host circulatory system at these early time points (P. Allen et al., 2013). After 3-4 days of implantation *in vivo*, however, human ECFC-derived vessels display robust connection with the host circulatory system (P. Allen et al., 2013). Previous studies have noted that up to 60% of human umbilical vein endothelial cells (HUVECs) undergo apoptosis in the first 24 hours following suspension in collagen matrices *in vitro* and there is further loss of vascular structures in 3D type I collagen matrices after 24 hours of culture (Ilan et al., 1998). Moreover, in our previous study, we reported that > 80% of human CB ECFC suspended in collagen matrices underwent apoptosis on day 1 after implantation (H. Kim, Huang, et al., 2015). After 2-3 days of implantation, only 1-3% of the total ECFC cells demonstrated viability in the subcutaneous implants (H. Kim, Huang, et al., 2015). Since the stability of newly formed vessels requires systemic blood flow, theses data suggest that implanted endothelial cells must survive at least 3-4 days *in vivo* to form and stabilize a capillary network that is connected to the host circulatory system.

Vasculogenesis, angiogenesis, and microvascular remodeling during embryonic development and in adult are complex and dynamic processes requiring endothelial cell growth, migration, and differentiation (Adams & Alitalo, 2007; Bader et al., 1998; Carmeliet, 2000; Carmeliet et al., 1996; Coultas et al., 2005;

Ferrara, 1999; Ferrara et al., 1996; Hanahan & Folkman, 1996; Lamalice et al., 2007; Risau, 1995, 1997; Shalaby et al., 1997). Human ECFC or HUVEC form a primary capillary network via cell-cell and/or cell-matrix interactions on 2D Matrigel coated dishes (Pollman et al., 1999). The formation of a capillary network is a highly dynamic process requiring both new network formation and concomitant network regression via apoptosis (Pollman et al., 1999). Thus, apoptosis of endothelial cells has been observed in the context of dynamic capillary network remodeling *in vitro* and *in vivo* and is thought to play a necessary role for optimal network formation (Brooks et al., 1994; Fukai et al., 1998; Pollman et al., 1999; Stratman et al., 2010). On the other hand, Bcl-2 over expression in HUVEC enhanced cell viability and promoted stabilization of the capillary network on Matrigel coated dishes. Therefore, the generation of anti-apoptotic signals is also required to maintain integrity of the vascular network following realignment of certain matrix proteins and cell-cell or cell-matrix interactions during the process (Pollman et al., 1999; Shih et al., 2011). Thus, understanding the balance between pro-apoptotic and pro-survival signals is a critical point in capillary structure formation and the remodeling process.

Apoptosis is a highly conserved cellular process in tissue development and homeostasis that generally culminates with the sequential activation of caspases (Duprez et al., 2009; Sprick & Walczak, 2004; Taylor et al., 2008). There are two pathways; the extrinsic pathway (death receptor pathway) and intrinsic pathway (mitochondrial pathway) that result in apoptosis depending on the apoptotic

signal. The extrinsic pathway is initiated by ligand binding to the death receptor family on the cell membrane, whereas the intrinsic pathway is triggered by stress-mediated damage such as alterations in temperature, osmolality, DNA damaging agents, free radical generation compounds, removal of nutrients, and oxygen deprivation (Duprez et al., 2009; Sprick & Walczak, 2004; Taylor et al., 2008; Wyllie, 2010). The activation of both pathways merges to generate the release of cytochrome c (Cyt-C) from the mitochondria and activation of the caspase cascade including executioner (effector) caspase-3 (Duprez et al., 2009; Sprick & Walczak, 2004; Taylor et al., 2008). Caspase-3 is activated to cleaved caspase-3 to generate all the biochemical and morphologic hallmarks of cell apoptosis (Duprez et al., 2009; Sprick & Walczak, 2004; Taylor et al., 2008). Caspases are a family of cytosolic aspartate-specific cysteine proteases involved in the initiation and execution of apoptosis (Duprez et al., 2009; Sprick & Walczak, 2004; Taylor et al., 2008). They are activated by an autoproteolytic mechanism or by cleavage by other proteases (frequently other caspases). We hypothesized that inhibition of caspases would increase ECFC survival and enhance ECFC vascularization in 3D collagen matrices.

4.2. Materials And Methods

Culture of human umbilical cord blood derived ECFC

ECFC were isolated and cultured as previously described (Ingram et al., 2004). ECFC colonies appeared between 5 and 22 days of culture and were indicated to form colonies of adherent cells with cobblestone morphology. The ECFC-derived

ECs were released from the culture plate by TrypLE™ Express (Gibco) and replated onto 25 or 75 cm^2 tissue culture flasks pre-coated with Type I rat-tail collagen (BD Biosciences) for subsequent passage after approximately 10-14 days of culture.

Two dimensional Matrigel assays *in vitro*

Tissue culture 96-well plates were coated with 50 µl of growth factor reduced Matrigel (BD Biosciences Pharmingen) and incubated for 30 minutes at 37°C, 5% CO_2. ECFC (1 x 10^4 cells) were resuspended in EGM-2 media with DMSO, Z-YVAD-FMK (0.5 mM, caspase-1/4 inhibitor), or Z-DEVD-FMK (0.5 mM, capase-3 inhibitor) and plated on Matrigel coated 96-well plates. The Z-YVAD-FMK and Z-DEVD-FMK were purchased from R&D system. ECFC plated on Matrigel was incubated for 24 hours, 48 hours and 72 hours. Images of tube-like structures were captured at 10x magnification after 24 hours, 48 hours, and 72 hours using a Leica DM IRE2 microscope (Leica Microsystems) with attached Retiga 4000R digital camera (QImaging).

Preparation of three dimensional (3D) collagen matrices assay

All of the type I collagen oligomers and associated polymerization reagents were purchased from GeniPhys. Zionsville, IN (Bailey et al., 2011). Stock oligomer was diluted in 0.01N hydrochloric acid (HCl) and neutralized according to manufacturer's recommendations to achieve a final oligomer concentration of 1.37 mg/ml (200Pa matrix stiffness). ECFC (1 x 10^5 cells/60 µl or 1 x 10^6

cells/250 µl) were suspended in the collagen solution with/without human platelet lysate (HPL-10% of final concentration), with DMSO vehicle (same amount of each inhibitor), Z-YVAD-FMK (0.5 mM, caspase-1/4 inhibitor), or Z-DEVD-FMK (0.5 mM, capase-3 inhibitor) at 4°C. The HPL was purchased from Gemeinnützige Salzburger Landeskliniken Betriebsges (SALK) in Graz, Austria. The HPL was prepared from pooled platelet-rich plasma derived from a minimum of 40 whole blood donations (Schallmoser & Strunk, 2009). The collagen-cell suspensions were plated on 96-well or 48-well plates, allowed to polymerize at 37°C for 30 minutes, and covered with complete endothelial cell growth medium (EGM-2, Lonza) with 10% defined fetal bovine serum (Hyclone) for incubation for one to three days in a tissue culture incubator at 37°C, 5% CO_2.

Toluidine blue staining of 3D collagen matrices and quantification of *in vitro* vascular structures

For analysis of *in vitro* vascular structure formation in 3D collagen matrix, cellularized collagen matrices were fixed with 4% paraformaldehyde for 20 minutes on Day 1 or Day 3 post-matrix formation. The matrices were stained with 0.1% toluidine blue O dye (30% methanol) for 25 minutes at room temperature and washed with PBS for 30 minutes for three times at room temperature (as previously described (Critser et al., 2010)). Vascular structures (Vacuoles, Lumens, and tube formation) were quantified using ImageJ image analysis software (National Institutes of Health (NIH)). Vacuoles and lumens were defined as areas completely surrounded by a toluidine blue stained endothelial cell

membrane. A single image of the entire well was captured at a depth of 100 microns from the surface of the matrix for each of triplicate samples of each group. Images of vascular structures were captured at 10x magnification using a Leica DM IRE2 microscope (Leica Microsystems) with attached Retiga 4000R digital camera (QImaging).

Assessment of apoptosis of ECFC in 3D collagen matrices

Apoptosis was assessed by examining the percentage of human $CD31^+$ ECFC that bound Annexin V and propidium iodide and was performed as per the manufacturer's instruction (Apoptosis Detection kit, eBioscience). ECFC (1×10^5 cells/60 µl) were suspended in collagen matrices with/without HPL, with DMSO vehicle (same volume of each inhibitor), Z-YVAD-FMK, or Z-DEVD-FMK, plated in 96-well plates, and incubated for one to three days as above. As previously described (H. Kim, Huang, et al., 2015), matrices were recovered from one to three days after implantation and incubated in 250 µl Collagenase Type I (0.25%) (Stemcell technology) for 20 minutes at 37°C. Cell dissociation buffer was added to stop the enzymatic reaction (Invitrogen). Cells were centrifuged at 500 g for 5 minutes at room temperature. Cell pellets were suspended in staining buffer and stained with anti-human CD31 antibody conjugated to phycoerythrin (PE) for 15 minutes (clone WM-59, BD Biosciences Pharmingen). Cells were incubated and stained for Annexin V-allophycocyanin (APC) and propidium iodide (PI) in binding buffer for 10 minutes at room temperature in the dark. Stained cells were analyzed by FlowJo software.

Western blot

Cellular protein lysates were extracted from the ECFC collagen matrix implants and electrophoresed using sodium dodecyl sulfate–polyacrylamide matrix electrophoresis (SDS-PAGE), transferred to nitrocellulose, and probed with monoclonal antibodies that included anti-human cleaved caspase-1 (clone D7F10, Cell signaling) and cleaved caspase-3 (clone 3G2, Cell signaling). Anti-human GAPDH (clone 14C10, Cell signaling) antibody was used to probe for loading control proteins (as previously described (H. Kim, Huang, et al., 2015)).

Histology and immunohistochemistry

Sections were stained as previously described (Yoder et al., 2007). Briefly, paraffin-embedded tissue sections were deparaffinized and then either directly stained with hematoxylin and eosin (H&E) or immersed in retrieval solution (Dako) for 20 minutes at 90–99°C. Slides were incubated at room temperature for 30 minutes with anti-human CD31 antibody (clone JC70/A, Abcam) followed by a 10-minute incubation with LASB2 link-biotin and streptavidin-HRP (Vector Laboratories), then developed with DAB (Vector Laboratories) solution for 5 minutes. Slides were analyzed by microscope under 20x magnification using a Leica DM 4000B microscope (Leica Microsystems, Bannockburn) with attached Spot-KE digital camera (Diagnostic Instruments, Sterling Heights).

Statistical analysis

Results are expressed as mean ± the standard error of the mean (SEM) for the study variables. The assessment of apoptosis of ECFC, *in vitro* vascular structure quantification and the quantification of western blot data points (ECFC treated with DMSO, Z-YVAD-FMK, or Z-DEVD-FMK) were assessed by unpaired t-test. A statistically significant difference was set at *P<0.05, **P<0.001, or ***P<0.0001.

4.3. Results

Caspase inhibitors reduce vasculogenesis of human CB derived ECFC on 2D Matrigel *in vitro*.

Consistent with prior reports, inhibition of programmed cell death significantly decreased vasculogenesis of endothelial cells (EC) via various types of caspase inhibitors (Rohban et al., 2013; Segura et al., 2002). We first examined whether the presence of Z-YVAD-FMK (0.5 mM, caspase-1/4 inhibitor) or Z-DEVD-FMK (0.5 mM, capase-3 inhibitor) alters ECFC vasculogenesis on 2D Matrigel coated dishes. The concentration (0.5 mM) of the caspase inhibitors was selected based upon two previous studies that reported these concentrations decreased vasculogenesis (Rohban et al., 2013; Segura et al., 2002). Both ECFC alone (Figure 4.1. a) and ECFC with the control solvent DMSO (Figure 4.1. D) formed tube-like structures on the Matrigel coating at 24 hours after cell deposition. The ECFC alone group formed and maintained the tube-like structures on the Matrigel coated dishes at 24, 48, and 72 hours of culture (Fig. 1A-C). Tube-like

structures of the ECFC with DMSO (vehicle control) group were slightly regressed through 48 and 72 hours of culture (Figure 4.1. D-F). However, the presence of caspase-1 inhibitor (0.5 mM) (Figure 4.1. G-I) and caspase-3 inhibitor (0.5 mM) (Figure 4.1. J-L) blocked the formation, elongation, and branching of tube-like structures during vasculogenesis of ECFC on 2D Matrigel coated dishes. The caspase-3 inhibitor group displayed the most dramatic capillary tube-like regression and loss of the tube-like structures over time. These data confirm that effector caspase inhibition in ECFC significantly impairs vasculogenesis of ECFC on 2D Matrigel coated dishes *in vitro* as recently reported (Rohban et al., 2013; Segura et al., 2002).

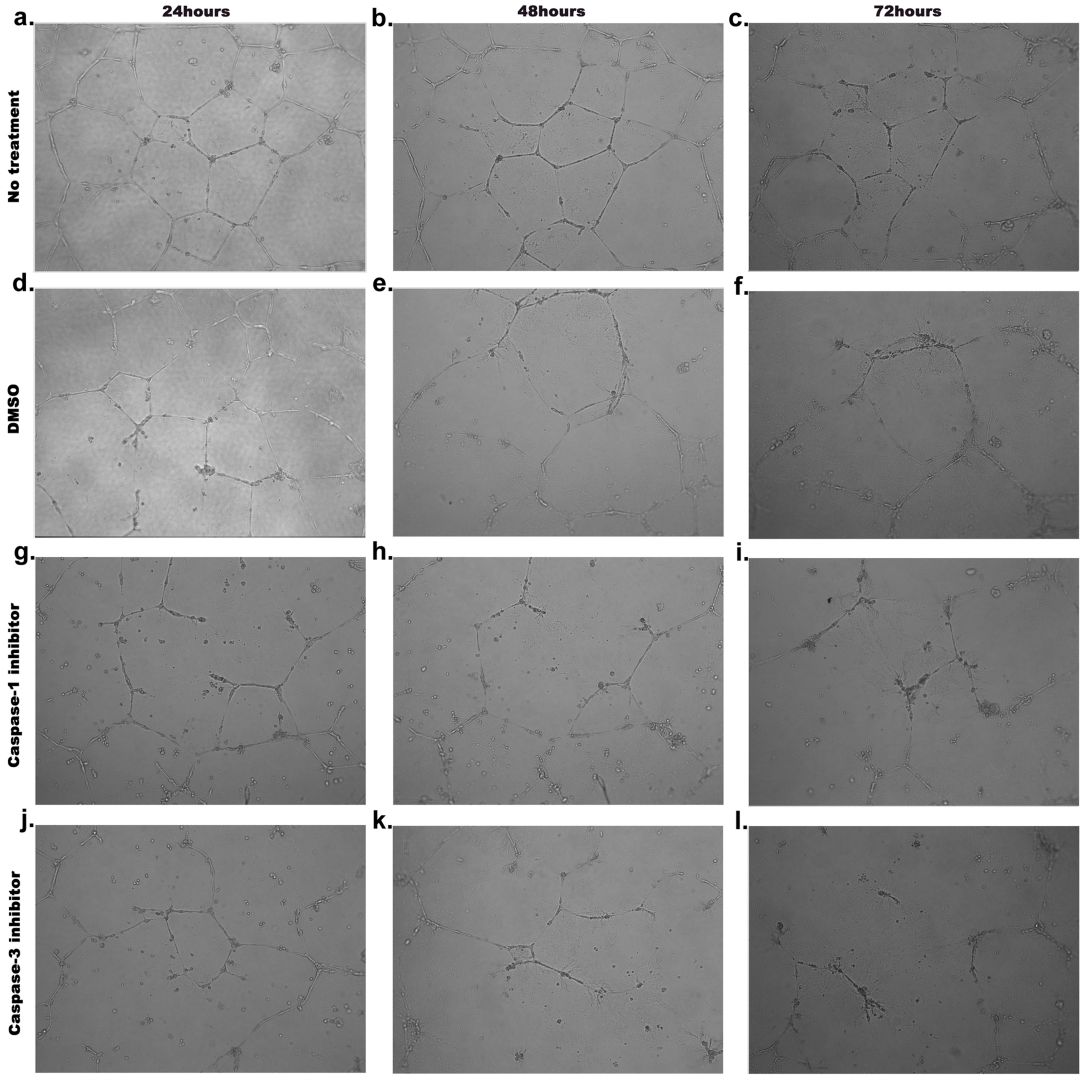


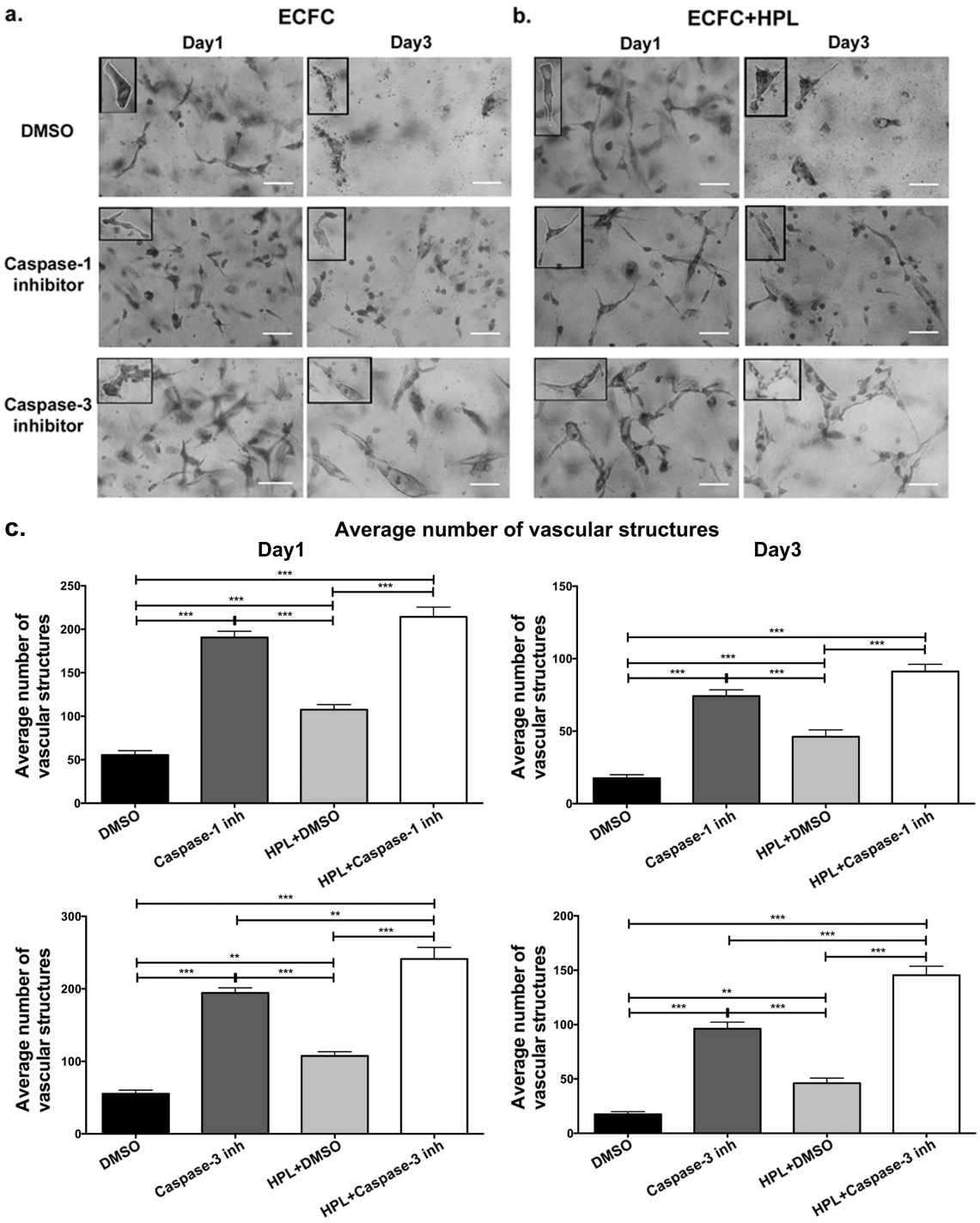
Figure 4.1. Inhibition of caspases in ECFC diminishes vasculogenic activity of ECFC on 2D Matrigel *in vitro*. The Images show representative images of tube-like structure formation of untreated control ECFC (a-c), ECFC with DMSO (d-f), with caspase-1 inhibitor (g-i), and with caspase-3 inhibitor (j-l).

Inhibition of caspase promotes vasculogenesis of human CB derived ECFC in 3D collagen matrices *in vitro*.

We next investigated whether co-implantation of ECFC with caspase inhibitors (same concentration as above with 2D Matrigel coated dishes) would also impair vasculogenic activity of ECFC in 3D collagen matrices. ECFC-derived vascular structures were defined as endothelial cells with vacuoles and lumenized structures were defined as areas completely surrounded by a toluidine blue stained endothelial cell membrane (Figure 4.2. a and b). A significantly greater average number of vascular structures per surface area of the matrix (Figure 4.2. c) and total luminal area of vascular structures per surface area of the matrix (mm^2) (Figure 4.2. d) were quantified in ECFC incubated with caspase-1 inhibitor and caspase-3 inhibitor compared to the control groups on day 1 and 3 of the *in vitro* study. Moreover, Caspase-3 inhibition displayed the greatest average number of vascular structures per surface area of the matrix and total luminal area of vascular structures per surface area of the matrix (mm^2) among the experimental groups on day 3 of culture.

We subsequently tested whether caspase inhibitors alter the protein expression level of cleaved caspase-1 or cleaved caspase-3 in ECFC in 3D collagen matrices *in vitro*. Cleaved caspases are the active forms that promote apoptosis. In the presence of caspase-3 inhibitor, cleaved caspase-3 was appropriately diminished in collagen gels containing ECFC alone and ECFC with added human platelet lysate (HPL) compared to the DMSO control group gels (Figure 4.2. e

and f). Inhibition of caspase-1 decreased cleaved caspase-1 in ECFC alone and ECFC with HPL group gels compared to DMSO containing gels (Figure 4.2. e and f). Thus, these data demonstrate that ECFC incubated with two selective caspase inhibitors significantly promotes vasculogenesis in 3D collagen matrices by blocking caspase-1 and -3 activity *in vitro*.


a.
ECFC
Day1
Day3
DMSO
Caspase-1 inhibitor
Caspase-3 inhibitor
b.
ECFC+HPL
Day1
Day3
c.
Average number of vascular structures
Day1
Day3
Average number of vascular structures
DMSO
Caspase-1 inh
HPL+DMSO
HPL+Caspase-1 inh
Caspase-3 inh
HPL+Caspase-3 inh

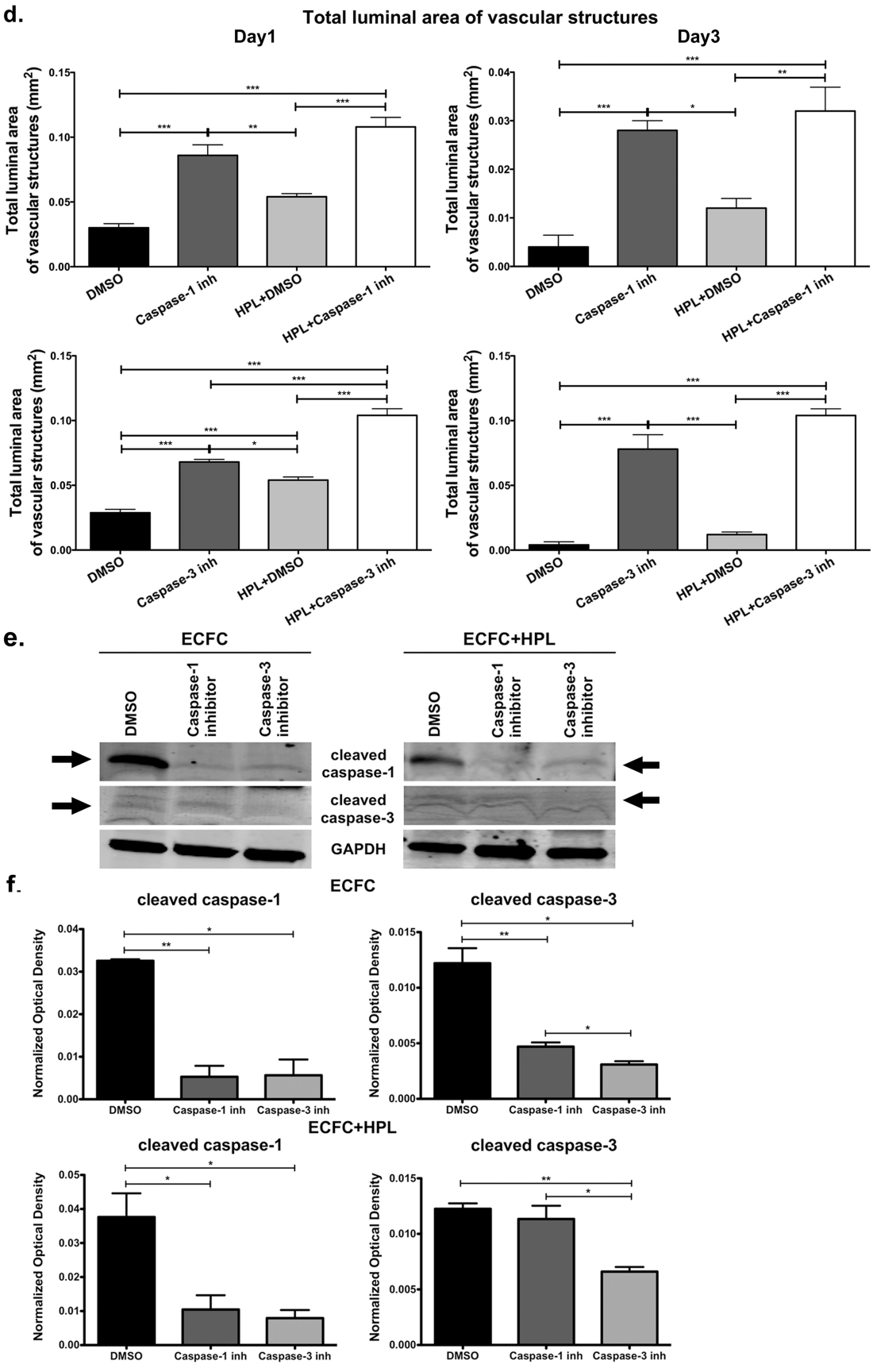
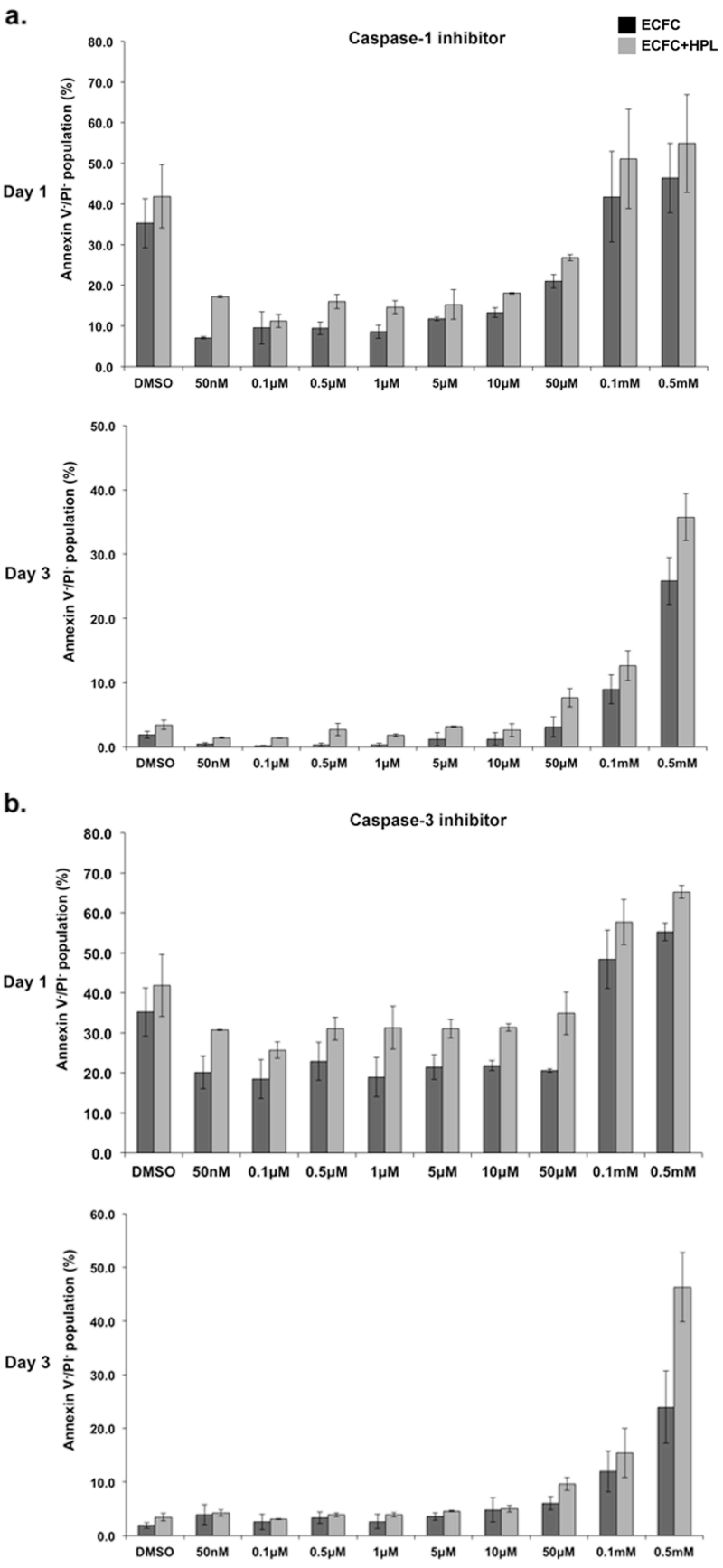
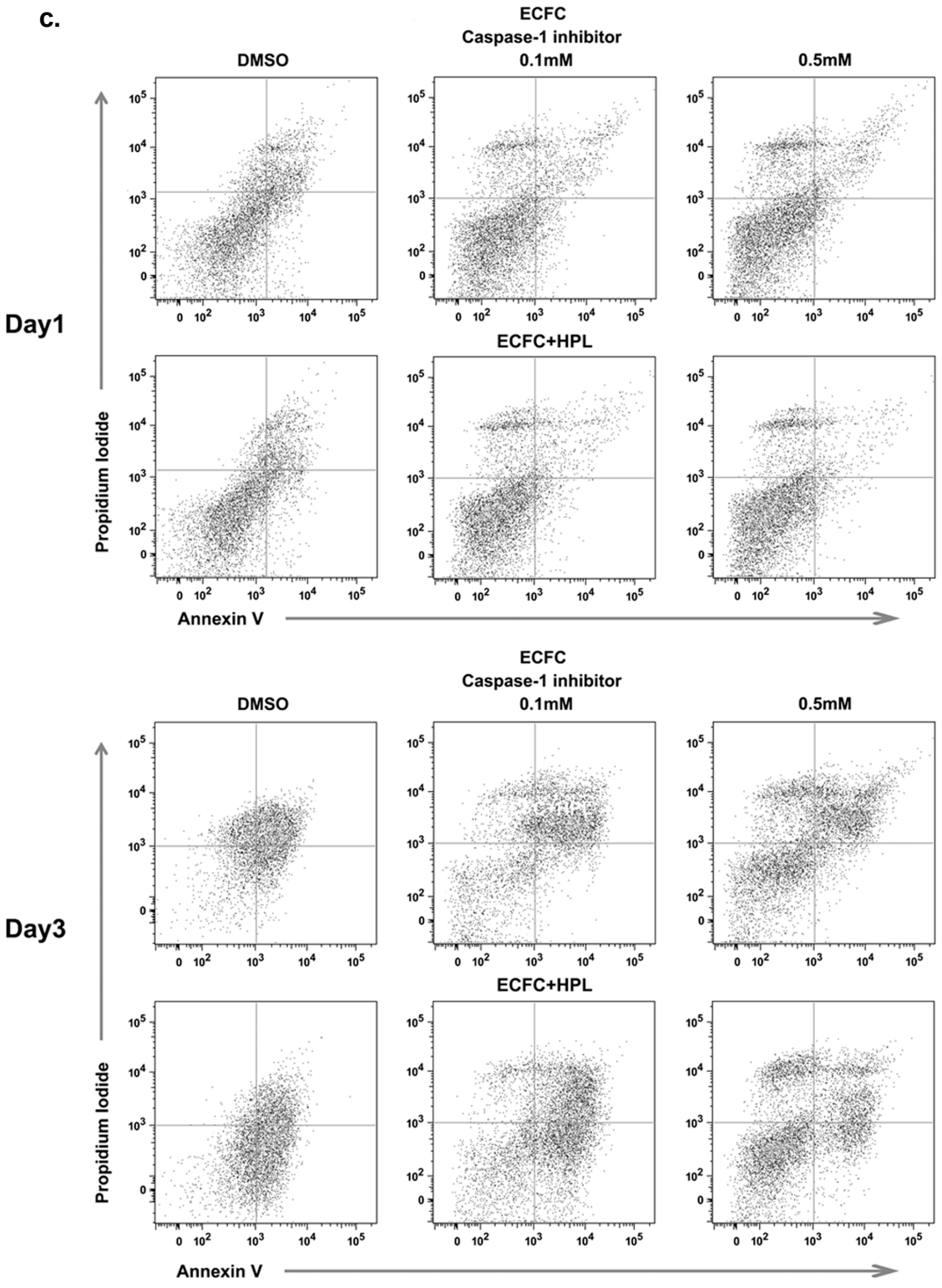

d.
Total luminal area of vascular structures
Day1
Day3
Total luminal area of vascular structures (mm2)
DMSO
Caspase-1 inh
HPL+DMSO
HPL+Caspase-1 inh
Caspase-3 inh
HPL+Caspase-3 inh
e.
ECFC
ECFC+HPL
DMSO
Caspase-1 inhibitor
Caspase-3 inhibitor
cleaved caspase-1
cleaved caspase-3
GAPDH
f.
ECFC
ECFC+HPL
cleaved caspase-1
cleaved caspase-3
Normalized Optical Density

Figure 4.2. ECFC with caspase inhibitors induces the formation of vascular structures in 3D collagen matrices *in vitro*. (a) Representative images of vascular structures of ECFC with or without HPL and with DMSO, caspase-1 inhibitor, or caspase-3 inhibitor in collagen matrices on day 1 and 3 (white arrows indicate the structures and small boxes indicates representative counted structures). Scale bar represents 0.05 μm. ECFC with caspase-1 inhibitor or caspase-3 inhibitor was resulted in a significant increase in (c) the average number of vascular structures per collagen matrix surface area and (d) total luminal area of vascular structures per matrix surface area (mm^2) compared to controls. N=10 each group. *P<0.05, **P<0.001, or ***P<0.0001. Top panel (e) displays representative immunoblots of cleaved caspase-1 and cleaved caspase-3 proteins from ECFC with DMSO, caspase-1 inhibitor, or caspase-3 inhibitor in 3D matrices. Bottom panel (f) shows quantification of repeated experiments on day 1 and day 3 after incubation (protein expression levels were normalized using GAPDH). N=2 each group. *P<0.05 or **P<0.001.

Human CB derived ECFC incubated with caspase inhibitors significantly enhanced cell survival of ECFC in 3D collagen matrices.

Our previous study demonstrated that activation of the Notch pathway by OP9-Delta like ligand 1 (DL1) stromal cells or co-implantation of ECFC with HPL enhances vasculogenesis and augments blood vessel formation by diminishing apoptosis of the implanted ECFC (H. Kim, Huang, et al., 2015). We subsequently tested whether the presence of caspase inhibitors diminishes apoptosis of ECFC in 3D collagen matrices. After 1-3 days, ECFCs were recovered from 3D collagen matrices and assessed for apoptosis of ECFC stained with anti-human CD31, Annexin V and propidium iodide (PI). The $CD31^+$ ECFC population was selected from the total cell population and separated into Annexin $V^{+/-}$ and $PI^{+/-}$ expressing subsets. The concentration of caspase-1 inhibitor and caspase-3 inhibitor in the 3D collagen matrices were titrated based on the percentage of viable cells (Annexin V^-/PI^-) (Figure 4.3.). ECFC incubated with DMSO started undergoing apoptosis on day 1 and a significant population of the cells died by day 3 in the collagen matrices *in vitro* (Figure 4.4.). In contrast, ECFC with added caspase-1 inhibitor or caspase-3 inhibitor revealed a significantly higher percentage of viable cells (Annexin V^-/PI^-) at the highest concentration of inhibitor (0.5 mM) compared to the control group. The addition of the caspase-3 inhibitor (0.5 mM) displayed the greatest percentage of viable cells compared to ECFC with added DMSO or caspase-1 inhibitor (0.5 mM). These data suggest significantly enhanced cell survival of ECFC in 3D collagen matrices *in vitro* in the presence of caspase-1 or -3 inhibitor.


a.
Caspase-1 inhibitor
ECFC
ECFC+HPL
Day 1
Annexin V-/PI- population (%)
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
DMSO
50nM
0.1μM
0.5μM
1μM
5μM
10μM
50μM
0.1mM
0.5mM
Day 3
Annexin V-/PI- population (%)
50.0
40.0
30.0
20.0
10.0
0.0
DMSO
50nM
0.1μM
0.5μM
1μM
5μM
10μM
50μM
0.1mM
0.5mM
b.
Caspase-3 inhibitor
Day 1
Annexin V-/PI- population (%)
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
DMSO
50nM
0.1μM
0.5μM
1μM
5μM
10μM
50μM
0.1mM
0.5mM
Day 3
Annexin V-/PI- population (%)
60.0
50.0
40.0
30.0
20.0
10.0
0.0
DMSO
50nM
0.1μM
0.5μM
1μM
5μM
10μM
50μM
0.1mM
0.5mM


c.
ECFC
Caspase-1 inhibitor
DMSO
0.1mM
0.5mM
Day1
ECFC+HPL
Propidium Iodide
Annexin V
ECFC
Caspase-1 inhibitor
DMSO
0.1mM
0.5mM
Day3
ECFC+HPL
Propidium Iodide
Annexin V

d.

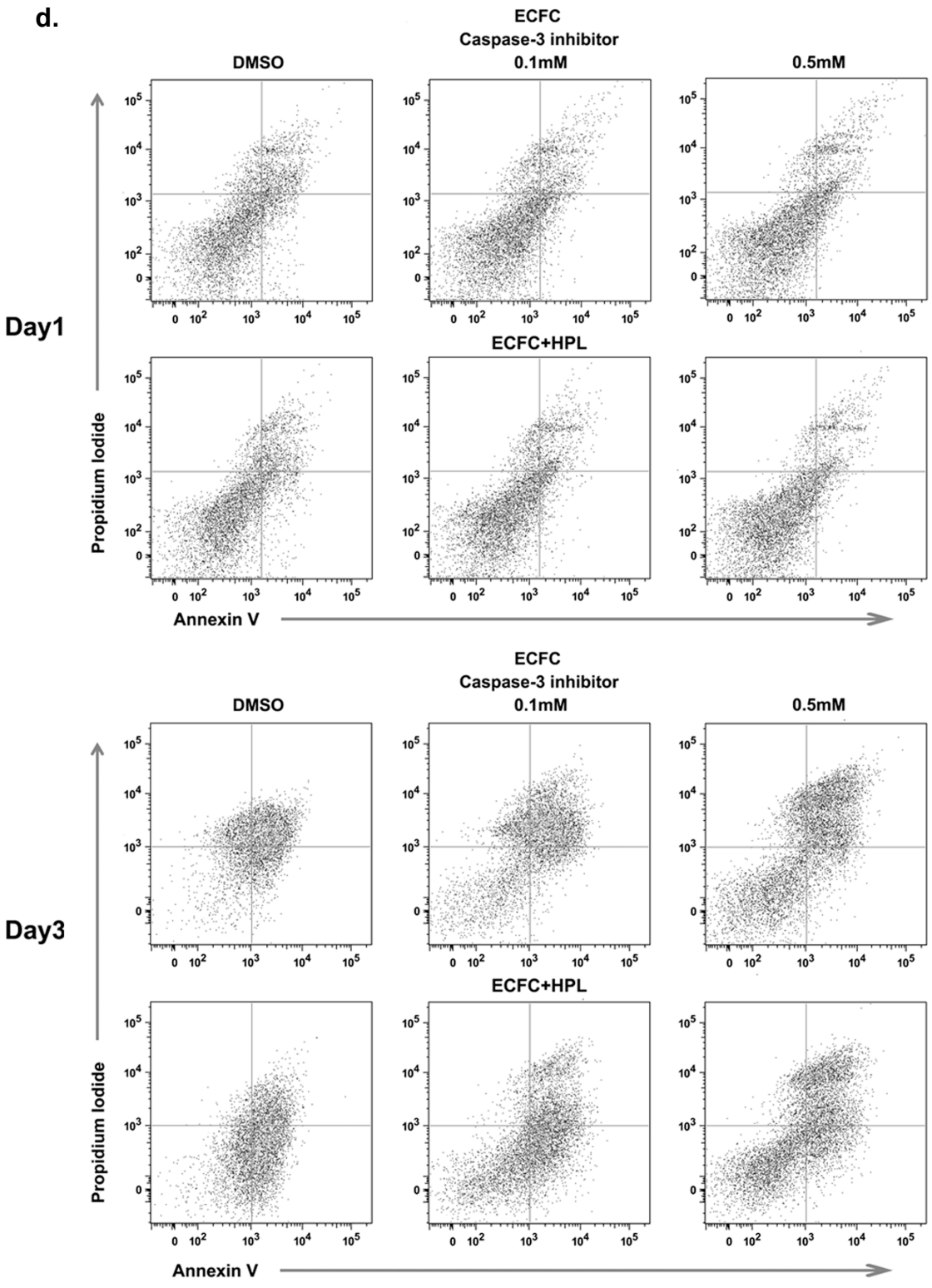

ECFC
Caspase-3 inhibitor
DMSO
0.1mM
0.5mM
Day1
ECFC+HPL
Propidium Iodide
Annexin V
ECFC
Caspase-3 inhibitor
DMSO
0.1mM
0.5mM
Day3
ECFC+HPL
Propidium Iodide
Annexin V

Figure 4.3. The effect of caspase inhibitors on ECFC in 3D collagen matrices *in vitro*. Percentage of the Annexin V^-/PI^- viable population indicates the dose-dependent inhibition of apoptosis in ECFC treated with DMSO, (a) caspase-1 inhibitor, or (b) caspase-3 inhibitor. (c and d) Representative dot plots of apoptosis analysis of ECFC with DMSO, caspase-1 inhibitor, or caspase-3 inhibitor on day 1 and 3 (only two concentrations of each inhibitor).

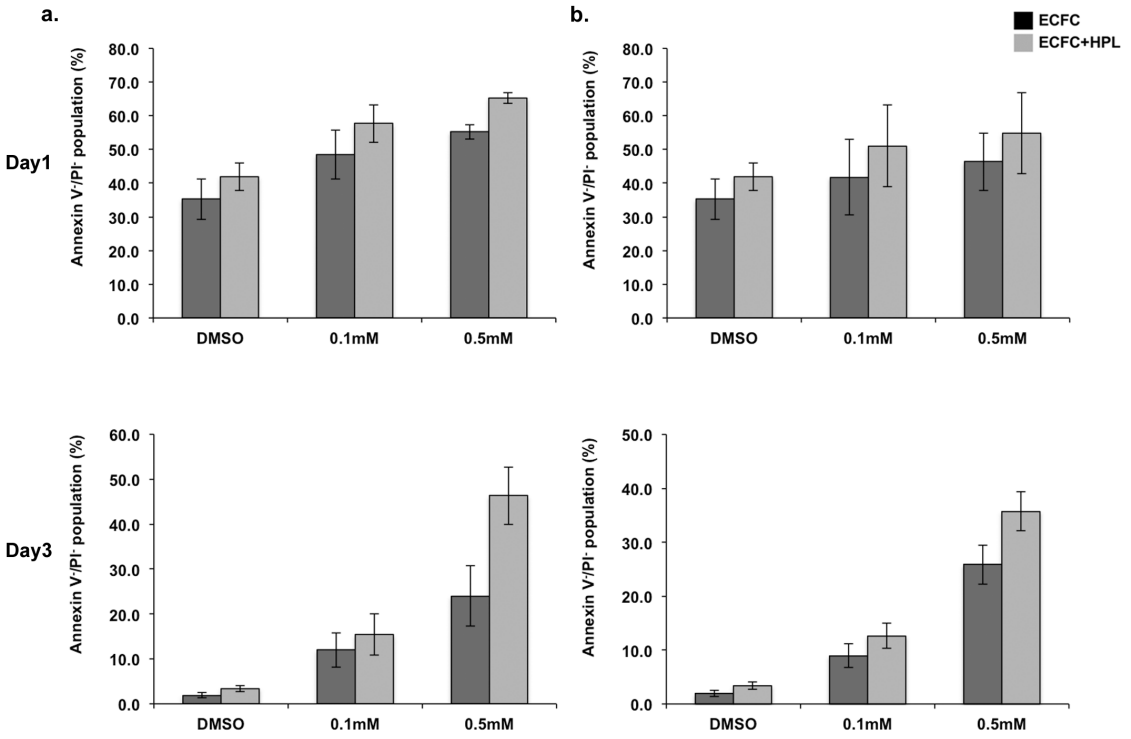


Figure 4.4. Incubation of ECFC with caspase inhibitors diminishes apoptosis of ECFC in 3D collagen matrices *in vitro*. The Annexin V^-/PI^- viable population of ECFC with (a) caspase-3 inhibitor and (b) caspase-1 inhibitor was significantly higher on day 1 and 3 compared to ECFC with DMSO in culture. N=3. *P<0.05.

4.4 Discussion

The balance between apoptosis and cell survival is an important variable during vasculogenesis, angiogenesis, and microvascular remodeling during embryonic development and in adult organisms (Jacobson, Weil, & Raff, 1997). Earlier reports showed that activation of apoptosis pathways by blocking cell-cell and/or cell-matrix interactions blocks vessel formation (Brooks et al., 1994; Fukai et al., 1998; Pollman et al., 1999; Stratman et al., 2010). Our previous studies also have demonstrated that activation of the Notch pathway by OP9-DL1 stromal cells or co-implantation of ECFC with HPL enhances vasculogenesis and augments blood vessel formation by diminishing apoptosis of the implanted ECFC. In contrast, some studies have been reported that caspase inhibition reduces adhesion and migration of endothelial cells (EC) and significantly decreases the vasculogenic activities of EC in a 2D Matrigel model (Rohban et al., 2013; Segura et al., 2002). Thus, the roles of apoptosis in vasculogenesis of EC are controversial and have not been well studied. The aim of this study was to examine whether inhibition of the apoptosis pathway via caspase inhibitors alters the early phase of vasculogenesis of ECFC when plated on 2D Matrigel or in 3D collagen matrices. When ECFC are plated on the 2D Matrigel, inhibition of activation of caspase-1 and caspase-3 significantly reduced vasculogenic activities of EC (Figure 4.1.), whereas ECFC with caspase-1 or caspase-3 inhibitors significantly increased vasculogenesis in 3D collagen matrices (Figure 4.2.).

In this report, we have provided evidence that 2D Matrigel and 3D collagen matrix environments revealed totally opposite results in ECFC survival and vasculogenic ability *in vitro*. At present we cannot determine whether the differences in ECFC survival and function were due to caspase inhibition in the context of the 2D or 3D environment or in response to the Matrigel or collagen matrix. For proper comparisons of each matrix model, in future studies we will examine cell survival and vasculogenesis of ECFC with caspase-1 and caspase-3 inhibitor in Matrigel and collagen matrices in both 2D and 3D formats.

We have previously published that non-human primate peripheral blood ECFC display vessel forming ability from newborn animals throughout adulthood *in vitro* and *in vivo* upon implantation in immunodeficient mice. Interestingly ECFC from those oldest animals failed to form non-human primate-derived ECFC-lined vessels when implanted *in vivo*, whereas the same ECFC formed capillary structures when plated *in vitro* on Matrigel coated dishes (Shelley et al., 2012). Thus, the responses of the ECFC derived from the oldest monkeys displayed differences in capillary tube formation depending on whether they were plated in a 2D Matrigel or a 3D collagen environment. Therefore, we cannot simply assume that *in vivo* vasculogenesis of ECFC will be promoted by inhibition of caspase-3, even though ECFC with caspase-3 inhibitor enhanced vasculogenesis in 3D collagen matrices *in vitro*. Thus, in future studies we will examine *in vivo* vessel forming ability of ECFC with caspase-3 inhibitor upon implantation in NOD/SCID mice.

CHAPTER 5. SUMMARY

In the adult organism, endothelial progenitor cells (EPCs) have been reported to contribute to neovascularization in the recovery of damaged vascular endothelium through the processes of angiogenesis and vasculogenesis (Asahara et al., 1997; Rafii et al., 2002; Shi et al., 1998). Reports over 5 decades have reported that some circulating mammalian ECs attach and proliferate to form colonies of endothelium that coat intravascular suspended biomaterials (Stump et al., 1963) while the majority of circulating human ECs are senescent sloughed cells undergoing anoikis (Woywodt et al., 2006). In 1997, Asahara reported that circulating endothelial progenitors could be isolated from human adult peripheral blood and participate in revascularization *in vivo* in response to acute tissue ischemia (Asahara et al., 1997). EPCs have been subsequently reported to contribute to new vessel formation in adult subjects (Asahara et al., 1997; Shi et al., 1998). For example, blood vessel damage leads to an increase in the number of circulating EPCs in patients with peripheral artery disease (PAD) (Hill et al., 2003; Rafii & Lyden, 2003; Urbich & Dimmeler, 2004).

Only certain subsets of circulating EPCs are recruited into areas of vascular injury to promote vascular repair (Urbich & Dimmeler, 2004; Werner et al., 2005). In immunodeficient mouse vascular injury models, human endothelial colony forming cells (ECFCs) integrate into injured vessels to relieve tissue ischemia (Schwarz et al., 2012) and have been reported to improve blood flow in various diseases, such as ischemic retinopathy, limb ischemia, myocardial infarction, and

stroke (X. T. Huang et al., 2013; Kang et al., 2013; Medina et al., 2010; Moubarik et al., 2011; Schwarz et al., 2012; Stroncek et al., 2012). Thus, ECFC may serve as a novel cell therapy for these disorders in human subjects.

We have isolated endothelial colony forming cells (ECFCs) from human peripheral blood and cord blood (Au, Daheron, et al., 2008; Ingram et al., 2004; Melero-Martin et al., 2007). ECFCs express cell surface proteins (KDR, CD34, vWF, eNOS, VE-cadherin, and others) similar to those on primary ECs (Ingram et al., 2004; Yoder et al., 2007). ECFCs have a high proliferative potential and are able to form human blood vessel de novo *in vivo* when implanted in immnunodeficient mice (Yoder et al., 2007). However, there is still a great need for further refinement of methods and identifying the molecular mechanisms that regulate and facilitate vascularization of ECFC in *in vitro* and *in vivo* matrices.

Human ECFC-derived vessels form after implantation in NOD/SCID mice, but inosculation to the host circulatory system does not occur until after 3-4 days of implantation (P. Allen et al., 2013). It has been known that up to 60% of human umbilical vein endothelial cells (HUVECs) undergo apoptosis in the first 24 hours after suspension in collagen gels (Ilan et al., 1998). We have also reported that most human CB ECFC suspended in 3D collagen matrices displayed apoptosis on day 1 post-implantation (H. Kim, Huang, et al., 2015). After 2-3 days of incubation, the majority of ECFC died. Only 1-3% of the total implanted cells were alive in the subcutaneous implants (H. Kim, Huang, et al., 2015). Since

systemic blood flow is required to stabilize newly formed vessels, these data suggest that implanted endothelial cells must survive at least 3-4 days *in vivo* to form a stable perfused capillary network that can be perfused by the host circulatory system.

Notch1 activation has been involved in inhibiting cell death in a cell type specific manner (Beverly et al., 2005; Jehn et al., 1999; MacKenzie et al., 2004; Sade et al., 2004). Notch 1 and a downstream mediator, Hes1, in human iliac artery endothelial cells (HIAECs) caused growth suppression but improved cell survival of the cultured cells. Also, activated Notch1 in HIAECs formed a more stabilized network and cord formation on Matrigel substrate in the presence of VEGF (Z. J. Liu et al., 2003). Delta like ligand 1 (Dll1)-dependent Notch signaling mediated by EphrinB2 has been reported to induce branching morphogenesis and network formation by human arterial endothelial cells (HAECs) plated on Matrigel (Limbourg et al., 2007). These reports have indicated that Notch1 signaling plays a role in regulating endothelial cell survival and network and cord formation *in vitro*. Since Notch signaling is a critical for embryonic blood vessel formation, we hypothesized that activation of Notch signaling in ECFC would enhance cultured ECFC vasculogenic abilities *in vitro* and *in vivo*. We report that preconditioning of ECFC with Notch activation is not sufficient to promote *in vivo* vasculogenesis, whereas co-implantation of ECFC with Notch ligand Delta-like 1 (DL1) expressing OP9 stromal cells (OP9-DL1) significantly increased vasculogenesis of ECFC by diminishing ECFC apoptosis *in vivo*.

Human platelet lysate (HPL) contains various cytokines and growth factors that are involved in the balance between apoptosis and cell survival in numerous cells involved in the repair of injured tissues (Freishtat et al., 2009; Gambim et al., 2007; Mause et al., 2010; Pakala et al., 1994; Sharron et al., 2012; Stellos & Gawaz, 2007). VEGF and angiopoietin-1 in HPL promote protein kinase B (Akt) activation that enhances cell survival and contributes to vascular development (Carmeliet et al., 1999; Fujio & Walsh, 1999; Gerber et al., 1998; I. Kim et al., 2000; Kontos et al., 1998; Papapetropoulos et al., 2000). Constitutive activation of the Akt/phosphoinositol-3-kinase (PI3K) pathway in endothelial cells stimulates cell survival of EC and promotes more vasculogenesis of ECs in serum free conditions (Seandel et al., 2008). Since platelets have a critical role in regulation of cell death and survival and contain granules filled with various growth factors and cytokines (Fekete et al., 2012; Shih et al., 2011), we hypothesized that HPL would diminish apoptosis of ECFC and promote ECFC vascularization by modulating the balance between apoptosis and cell survival of ECFC in 3D collagen matrices. We report that ECFC mixed with HPL in collagen matrices decreased apoptosis of ECFC by altering the expression of pro-survival molecules (pAkt1, pBad, and Bcl-xL) *in vitro* and stimulated vasculogenesis of human EC-derived vessels both *in vitro* and *in vivo*.

Apoptosis is a highly conserved cellular process in tissue development and homeostasis that generally culminates with the sequential activation of effector caspases (Duprez et al., 2009; Sprick & Walczak, 2004; Taylor et al., 2008).

There are two pathways; the extrinsic pathway (death receptor pathway) and intrinsic pathway (mitochondrial pathway) that result in apoptosis depending on the apoptotic signal. The activation of both pathways leads to release of cytochrome c (Cyt-C) from mitochondria and activation of the executioner caspase-3 (Duprez et al., 2009; Sprick & Walczak, 2004; Taylor et al., 2008). Caspase-3 is activated to cleaved caspase-3 that generates all the biochemical and morphologic hallmarks of cell apoptosis (Duprez et al., 2009; Sprick & Walczak, 2004; Taylor et al., 2008). However, numerous reports have highlighted that capillary tube formation by EC plated on Matrigel *in vitro* is dependent on some level of caspase activation and apoptosis induction (Rohban et al., 2013; Segura et al., 2002). We hypothesized that inhibition of apoptosis via caspase inhibitors would increase ECFC survival and enhance ECFC vascularization in 3D collagen matrices *in vitro*. We report that inhibition of caspase-1 and -3 in ECFC significantly enhanced cell survival of ECFC and dramatically increased vascular formation of ECFC by decreasing cleaved caspase-1 and cleaved caspase-3 in 3D collagen matrices *in vitro*.

We hypothesized that *in vivo* vasculogenesis of ECFC would be enhanced by inhibition of caspase-3, since ECFC with caspase-3 inhibitor promoted the most vasculogenesis in 3D collagen matrices *in vitro*. We examined *in vivo* vessel forming ability of ECFC with caspase-3 inhibitor upon implantation in NOD/SCID mice. The inhibitor was injected into an implanted osmotic pump for constant delivery for 3 days post-implantation. The first set of mice receiving implants

showed increased vessel forming ability of ECFC in implants that included caspase-3 inhibitor compared to ECFC implants with DMSO control. However, in the second experiment, no difference in vessel forming ability of ECFC was observed in the two study groups. Kinetics of caspase-3 inhibitor release from the osmotic pump has not been well studied following implantation in mice. Further studies will be required to define kinetics of release of the caspase-3 inhibitor *in vivo* before repeating the ECFC implants studies.

In future studies, we can also examine combinational treatment of caspase-3 inhibitor with other drugs. For example, since ECFCs are in a hypoxic microenvironment following implantation, pre-conditioning of ECFC with hypoxia-inducible factor (HIF) inducers might lead to hypoxia tolerance to enhance cell survival of ECFC *in vivo*.

REFERENCES

Aarabi, S., Longaker, M. T., & Gurtner, G. C. (2007). Hypertrophic scar formation following burns and trauma: new approaches to treatment. *PLoS Med, 4*(9), e234. doi: 10.1371/journal.pmed.0040234

Ables, J. L., Breunig, J. J., Eisch, A. J., & Rakic, P. (2011). Not(ch) just development: Notch signalling in the adult brain. *Nat Rev Neurosci, 12*(5), 269-283. doi: nrn3024 [pii]10.1038/nrn3024

Achen, M. G., Jeltsch, M., Kukk, E., Makinen, T., Vitali, A., Wilks, A. F., . . . Stacker, S. A. (1998). Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). *Proc Natl Acad Sci U S A, 95*(2), 548-553.

Adams, R. H., & Alitalo, K. (2007). Molecular regulation of angiogenesis and lymphangiogenesis. *Nat Rev Mol Cell Biol, 8*(6), 464-478. doi: nrm2183 [pii]10.1038/nrm2183

Allen, P., Kang, K.-T., & Bischoff, J. (2013). Rapid onset of perfused blood vessels after implantation of ECFCs and MPCs in collagen, PuraMatrix and fibrin provisional matrices. *Journal of Tissue Engineering and Regenerative Medicine*, n/a-n/a. doi: 10.1002/term.1803

Allen, P., Kang, K. T., & Bischoff, J. (2013). Rapid onset of perfused blood vessels after implantation of ECFCs and MPCs in collagen, PuraMatrix and fibrin provisional matrices. *J Tissue Eng Regen Med*. doi: 10.1002/term.1803

Allen, P., Melero-Martin, J., & Bischoff, J. (2011). Type I collagen, fibrin and PuraMatrix matrices provide permissive environments for human endothelial and mesenchymal progenitor cells to form neovascular networks. *J Tissue Eng Regen Med, 5*(4), e74-86. doi: 10.1002/term.389

Alva, J. A., & Iruela-Arispe, M. L. (2004). Notch signaling in vascular morphogenesis. *Curr Opin Hematol, 11*(4), 278-283.

Amaya, E., Musci, T. J., & Kirschner, M. W. (1991). Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in Xenopus embryos. *Cell, 66*(2), 257-270.

Aplin, A. C., Fogel, E., Zorzi, P., & Nicosia, R. F. (2008). The aortic ring model of angiogenesis. *Methods Enzymol, 443*, 119-136. doi: 10.1016/S0076-6879(08)02007-7

Aranguren, X. L., Luttun, A., Clavel, C., Moreno, C., Abizanda, G., Barajas, M. A., . . . Prosper, F. (2007). In vitro and in vivo arterial differentiation of human multipotent adult progenitor cells. *Blood, 109*(6), 2634-2642. doi: blood-2006-06-030411 [pii]10.1182/blood-2006-06-030411

Asahara, T., Murohara, T., Sullivan, A., Silver, M., van der Zee, R., Li, T., . . . Isner, J. M. (1997). Isolation of putative progenitor endothelial cells for angiogenesis. *Science, 275*(5302), 964-967.

Au, P., Daheron, L. M., Duda, D. G., Cohen, K. S., Tyrrell, J. A., Lanning, R. M., . . . Jain, R. K. (2008). Differential in vivo potential of endothelial progenitor cells from human umbilical cord blood and adult peripheral blood to form

functional long-lasting vessels. *Blood, 111*(3), 1302-1305. doi: blood-2007-06-094318 [pii]10.1182/blood-2007-06-094318

Au, P., Tam, J., Fukumura, D., & Jain, R. K. (2008). Bone marrow-derived mesenchymal stem cells facilitate engineering of long-lasting functional vasculature. *Blood, 111*(9), 4551-4558. doi: blood-2007-10-118273 [pii] 10.1182/blood-2007-10-118273

Auguste, P., Gursel, D. B., Lemiere, S., Reimers, D., Cuevas, P., Carceller, F., . . . Bikfalvi, A. (2001). Inhibition of fibroblast growth factor/fibroblast growth factor receptor activity in glioma cells impedes tumor growth by both angiogenesis-dependent and -independent mechanisms. *Cancer Res, 61*(4), 1717-1726.

Bader, B. L., Rayburn, H., Crowley, D., & Hynes, R. O. (1998). Extensive vasculogenesis, angiogenesis, and organogenesis precede lethality in mice lacking all alpha v integrins. *Cell, 95*(4), 507-519.

Bailey, J. L., Critser, P. J., Whittington, C., Kuske, J. L., Yoder, M. C., & Voytik-Harbin, S. L. (2011). Collagen oligomers modulate physical and biological properties of three-dimensional self-assembled matrices. *Biopolymers, 95*(2), 77-93. doi: 10.1002/bip.21537

Barry, O. P., Pratico, D., Savani, R. C., & FitzGerald, G. A. (1998). Modulation of monocyte-endothelial cell interactions by platelet microparticles. *J Clin Invest, 102*(1), 136-144. doi: 10.1172/JCI2592

Bayless, K. J., & Davis, G. E. (2002). The Cdc42 and Rac1 GTPases are required for capillary lumen formation in three-dimensional extracellular matrices. *J Cell Sci, 115*(Pt 6), 1123-1136.

Bayless, K. J., & Davis, G. E. (2003). Sphingosine-1-phosphate markedly induces matrix metalloproteinase and integrin-dependent human endothelial cell invasion and lumen formation in three-dimensional collagen and fibrin matrices. *Biochem Biophys Res Commun, 312*(4), 903-913.

Bayless, K. J., Salazar, R., & Davis, G. E. (2000). RGD-dependent vacuolation and lumen formation observed during endothelial cell morphogenesis in three-dimensional fibrin matrices involves the alpha(v)beta(3) and alpha(5)beta(1) integrins. *Am J Pathol, 156*(5), 1673-1683.

Beckers, J., Clark, A., Wunsch, K., Hrabe De Angelis, M., & Gossler, A. (1999). Expression of the mouse Delta1 gene during organogenesis and fetal development. *Mech Dev, 84*(1-2), 165-168.

Bendinelli, P., Matteucci, E., Dogliotti, G., Corsi, M. M., Banfi, G., Maroni, P., & Desiderio, M. A. (2010). Molecular basis of anti-inflammatory action of platelet-rich plasma on human chondrocytes: mechanisms of NF-kappaB inhibition via HGF. *J Cell Physiol, 225*(3), 757-766. doi: 10.1002/jcp.22274

Benedito, R., Roca, C., Sorensen, I., Adams, S., Gossler, A., Fruttiger, M., & Adams, R. H. (2009). The notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. *Cell, 137*(6), 1124-1135. doi: 10.1016/j.cell.2009.03.025

Benedito, R., Trindade, A., Hirashima, M., Henrique, D., da Costa, L. L., Rossant, J., . . . Duarte, A. (2008). Loss of Notch signalling induced by Dll4 causes

arterial calibre reduction by increasing endothelial cell response to angiogenic stimuli. *BMC Dev Biol, 8*, 117. doi: 1471-213X-8-117 [pii] 10.1186/1471-213X-8-117

Beverly, L. J., Felsher, D. W., & Capobianco, A. J. (2005). Suppression of p53 by Notch in lymphomagenesis: implications for initiation and regression. *Cancer Res, 65*(16), 7159-7168. doi: 10.1158/0008-5472.CAN-05-1664

Boyer-Di Ponio, J., El-Ayoubi, F., Glacial, F., Ganeshamoorthy, K., Driancourt, C., Godet, M., . . . Uzan, G. (2014). Instruction of circulating endothelial progenitors in vitro towards specialized blood-brain barrier and arterial phenotypes. *PLoS One, 9*(1), e84179. doi: 10.1371/journal.pone.0084179

Breier, G., Albrecht, U., Sterrer, S., & Risau, W. (1992). Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. *Development, 114*(2), 521-532.

Breier, G., & Risau, W. (1996). The role of vascular endothelial growth factor in blood vessel formation. *Trends Cell Biol, 6*(12), 454-456.

Brooks, P. C., Montgomery, A. M., Rosenfeld, M., Reisfeld, R. A., Hu, T., Klier, G., & Cheresh, D. A. (1994). Integrin alpha v beta 3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell, 79*(7), 1157-1164.

Carmeliet, P. (2000). Mechanisms of angiogenesis and arteriogenesis. *Nat Med, 6*(4), 389-395. doi: 10.1038/74651

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., . . . Nagy, A. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature, 380*(6573), 435-439. doi: 10.1038/380435a0

Carmeliet, P., Lampugnani, M. G., Moons, L., Breviario, F., Compernolle, V., Bono, F., . . . Dejana, E. (1999). Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell, 98*(2), 147-157.

Chen, W. S., Xu, P. Z., Gottlob, K., Chen, M. L., Sokol, K., Shiyanova, T., . . . Hay, N. (2001). Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. *Genes Dev, 15*(17), 2203-2208. doi: 10.1101/gad.913901

Chen, Y., Jacamo, R., Shi, Y. X., Wang, R. Y., Battula, V. L., Konoplev, S., . . . Andreeff, M. (2012). Human extramedullary bone marrow in mice: a novel in vivo model of genetically controlled hematopoietic microenvironment. *Blood, 119*(21), 4971-4980. doi: 10.1182/blood-2011-11-389957

Cheng, G., Liao, S., Kit Wong, H., Lacorre, D. A., di Tomaso, E., Au, P., . . . Munn, L. L. (2011). Engineered blood vessel networks connect to host vasculature via wrapping-and-tapping anastomosis. *Blood, 118*(17), 4740-4749. doi: 10.1182/blood-2011-02-338426

Copeland, J. N., Feng, Y., Neradugomma, N. K., Fields, P. E., & Vivian, J. L. (2011). Notch signaling regulates remodeling and vessel diameter in the extraembryonic yolk sac. *BMC Dev Biol, 11*, 12. doi: 1471-213X-11-12 [pii] 10.1186/1471-213X-11-12

Cornell, R. A., & Kimelman, D. (1994a). Activin-mediated mesoderm induction requires FGF. *Development, 120*(2), 453-462.

Cornell, R. A., & Kimelman, D. (1994b). Combinatorial signaling in development. *Bioessays, 16*(8), 577-581. doi: 10.1002/bies.950160811

Coultas, L., Chawengsaksophak, K., & Rossant, J. (2005). Endothelial cells and VEGF in vascular development. *Nature, 438*(7070), 937-945. doi: 10.1038/nature04479

Critser, P. J., Kreger, S. T., Voytik-Harbin, S. L., & Yoder, M. C. Collagen matrix physical properties modulate endothelial colony forming cell-derived vessels in vivo. *Microvasc Res, 80*(1), 23-30. doi: S0026-2862(10)00046-4 [pii]10.1016/j.mvr.2010.03.001

Critser, P. J., Kreger, S. T., Voytik-Harbin, S. L., & Yoder, M. C. (2010). Collagen matrix physical properties modulate endothelial colony forming cell-derived vessels in vivo. *Microvasc Res, 80*(1), 23-30. doi: 10.1016/j.mvr.2010.03.001

Crowley, S. T., Dempsey, E. C., Horwitz, K. B., & Horwitz, L. D. (1994). Platelet-induced vascular smooth muscle cell proliferation is modulated by the growth amplification factors serotonin and adenosine diphosphate. *Circulation, 90*(4), 1908-1918.

Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., & Greenberg, M. E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell, 91*(2), 231-241.

Davis, G. E., Bayless, K. J., & Mavila, A. (2002). Molecular basis of endothelial cell morphogenesis in three-dimensional extracellular matrices. *Anat Rec, 268*(3), 252-275. doi: 10.1002/ar.10159

Davis, G. E., & Camarillo, C. W. (1996). An alpha 2 beta 1 integrin-dependent pinocytic mechanism involving intracellular vacuole formation and coalescence regulates capillary lumen and tube formation in three-dimensional collagen matrix. *Exp Cell Res, 224*(1), 39-51. doi: 10.1006/excr.1996.0109

Davis, G. E., Koh, W., & Stratman, A. N. (2007). Mechanisms controlling human endothelial lumen formation and tube assembly in three-dimensional extracellular matrices. *Birth Defects Res C Embryo Today, 81*(4), 270-285. doi: 10.1002/bdrc.20107

Davis, G. E., & Senger, D. R. (2005). Endothelial extracellular matrix: biosynthesis, remodeling, and functions during vascular morphogenesis and neovessel stabilization. *Circ Res, 97*(11), 1093-1107. doi: 10.1161/01.RES.0000191547.64391.e3

de Boer, H. C., Verseyden, C., Ulfman, L. H., Zwaginga, J. J., Bot, I., Biessen, E. A., . . . van Zonneveld, A. J. (2006). Fibrin and activated platelets cooperatively guide stem cells to a vascular injury and promote differentiation towards an endothelial cell phenotype. *Arterioscler Thromb Vasc Biol, 26*(7), 1653-1659. doi: 10.1161/01.ATV.0000222982.55731.f1

De Moerlooze, L., Spencer-Dene, B., Revest, J. M., Hajihosseini, M., Rosewell, I., & Dickson, C. (2000). An important role for the IIIb isoform of fibroblast

growth factor receptor 2 (FGFR2) in mesenchymal-epithelial signalling during mouse organogenesis. *Development, 127*(3), 483-492.

de Vries, C., Escobedo, J. A., Ueno, H., Houck, K., Ferrara, N., & Williams, L. T. (1992). The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science, 255*(5047), 989-991.

Delaney, C., Varnum-Finney, B., Aoyama, K., Brashem-Stein, C., & Bernstein, I. D. (2005). Dose-dependent effects of the Notch ligand Delta1 on ex vivo differentiation and in vivo marrow repopulating ability of cord blood cells. *Blood, 106*(8), 2693-2699. doi: 2005-03-1131 [pii]10.1182/blood-2005-03-1131

Deng, C. X., Wynshaw-Boris, A., Shen, M. M., Daugherty, C., Ornitz, D. M., & Leder, P. (1994). Murine FGFR-1 is required for early postimplantation growth and axial organization. *Genes Dev, 8*(24), 3045-3057.

Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R., & Zeiher, A. M. (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature, 399*(6736), 601-605. doi: 10.1038/21224

Djonov, V., & Makanya, A. N. (2005). New insights into intussusceptive angiogenesis. *EXS*(94), 17-33.

Duarte, A., Hirashima, M., Benedito, R., Trindade, A., Diniz, P., Bekman, E., . . . Rossant, J. (2004). Dosage-sensitive requirement for mouse Dll4 in artery development. *Genes Dev, 18*(20), 2474-2478. doi: 10.1101/gad.1239004

Dummler, B., Tschopp, O., Hynx, D., Yang, Z. Z., Dirnhofer, S., & Hemmings, B. A. (2006). Life with a single isoform of Akt: mice lacking Akt2 and Akt3 are viable but display impaired glucose homeostasis and growth deficiencies. *Mol Cell Biol, 26*(21), 8042-8051. doi: 10.1128/MCB.00722-06

Dumont, D. J., Fong, G. H., Puri, M. C., Gradwohl, G., Alitalo, K., & Breitman, M. L. (1995). Vascularization of the mouse embryo: a study of flk-1, tek, tie, and vascular endothelial growth factor expression during development. *Dev Dyn, 203*(1), 80-92. doi: 10.1002/aja.1002030109

Duprez, L., Wirawan, E., Vanden Berghe, T., & Vandenabeele, P. (2009). Major cell death pathways at a glance. *Microbes Infect, 11*(13), 1050-1062. doi: 10.1016/j.micinf.2009.08.013

Eichmann, A., Marcelle, C., Breant, C., & Le Douarin, N. M. (1993). Two molecules related to the VEGF receptor are expressed in early endothelial cells during avian embryonic development. *Mech Dev, 42*(1-2), 33-48.

El-Sharkawy, H., Kantarci, A., Deady, J., Hasturk, H., Liu, H., Alshahat, M., & Van Dyke, T. E. (2007). Platelet-rich plasma: growth factors and pro- and anti-inflammatory properties. *J Periodontol, 78*(4), 661-669. doi: 10.1902/jop.2007.060302

Enis, D. R., Shepherd, B. R., Wang, Y., Qasim, A., Shanahan, C. M., Weissberg, P. L., . . . Schechner, J. S. (2005). Induction, differentiation, and remodeling of blood vessels after transplantation of Bcl-2-transduced endothelial cells. *Proc Natl Acad Sci U S A, 102*(2), 425-430. doi: 0408357102 [pii]10.1073/pnas.0408357102

Eswarakumar, V. P., Monsonego-Ornan, E., Pines, M., Antonopoulou, I., Morriss-Kay, G. M., & Lonai, P. (2002). The IIIc alternative of Fgfr2 is a positive regulator of bone formation. *Development, 129*(16), 3783-3793.

Fekete, N., Gadelorge, M., Furst, D., Maurer, C., Dausend, J., Fleury-Cappellesso, S., . . . Rojewski, M. T. (2012). Platelet lysate from whole blood-derived pooled platelet concentrates and apheresis-derived platelet concentrates for the isolation and expansion of human bone marrow mesenchymal stromal cells: production process, content and identification of active components. *Cytotherapy, 14*(5), 540-554. doi: 10.3109/14653249.2012.655420

Feldman, B., Poueymirou, W., Papaioannou, V. E., DeChiara, T. M., & Goldfarb, M. (1995). Requirement of FGF-4 for postimplantation mouse development. *Science, 267*(5195), 246-249.

Ferrara, N. (1996). Vascular endothelial growth factor. *Eur J Cancer, 32A*(14), 2413-2422.

Ferrara, N. (1999). Role of vascular endothelial growth factor in the regulation of angiogenesis. *Kidney Int, 56*(3), 794-814. doi: 10.1046/j.1523-1755.1999.00610.x

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K. S., . . . Moore, M. W. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature, 380*(6573), 439-442. doi: 10.1038/380439a0

Flamme, I., Breier, G., & Risau, W. (1995). Vascular endothelial growth factor (VEGF) and VEGF receptor 2 (flk-1) are expressed during vasculogenesis and vascular differentiation in the quail embryo. *Dev Biol, 169*(2), 699-712. doi: 10.1006/dbio.1995.1180

Flamme, I., & Risau, W. (1992). Induction of vasculogenesis and hematopoiesis in vitro. *Development, 116*(2), 435-439.

Fong, G. H., Rossant, J., Gertsenstein, M., & Breitman, M. L. (1995). Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature, 376*(6535), 66-70. doi: 10.1038/376066a0

Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., . . . Tsichlis, P. N. (1995). The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell, 81*(5), 727-736.

Freishtat, R. J., Natale, J., Benton, A. S., Cohen, J., Sharron, M., Wiles, A. A., . . . Hoffman, E. P. (2009). Sepsis alters the megakaryocyte-platelet transcriptional axis resulting in granzyme B-mediated lymphotoxicity. *Am J Respir Crit Care Med, 179*(6), 467-473. doi: 10.1164/rccm.200807-1085OC

Fujio, Y., Nguyen, T., Wencker, D., Kitsis, R. N., & Walsh, K. (2000). Akt promotes survival of cardiomyocytes in vitro and protects against ischemia-reperfusion injury in mouse heart. *Circulation, 101*(6), 660-667.

Fujio, Y., & Walsh, K. (1999). Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner. *J Biol Chem, 274*(23), 16349-16354.

Fukai, F., Mashimo, M., Akiyama, K., Goto, T., Tanuma, S., & Katayama, T. (1998). Modulation of apoptotic cell death by extracellular matrix proteins and a fibronectin-derived antiadhesive peptide. *Exp Cell Res, 242*(1), 92-99.

Fulton, D., Gratton, J. P., McCabe, T. J., Fontana, J., Fujio, Y., Walsh, K., . . . Sessa, W. C. (1999). Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature, 399*(6736), 597-601. doi: 10.1038/21218

Gabler, C., Plath-Gabler, A., Killian, G. J., Berisha, B., & Schams, D. (2004). Expression pattern of fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) system members in bovine corpus luteum endothelial cells during treatment with FGF-2, VEGF or oestradiol. *Reprod Domest Anim, 39*(5), 321-327. doi: 10.1111/j.1439-0531.2004.00517.x

Gale, N. W., Dominguez, M. G., Noguera, I., Pan, L., Hughes, V., Valenzuela, D. M., . . . Yancopoulos, G. D. (2004). Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. *Proc Natl Acad Sci U S A, 101*(45), 15949-15954. doi: 0407290101 [pii]10.1073/pnas.0407290101

Gambim, M. H., do Carmo Ade, O., Marti, L., Verissimo-Filho, S., Lopes, L. R., & Janiszewski, M. (2007). Platelet-derived exosomes induce endothelial cell apoptosis through peroxynitrite generation: experimental evidence for a novel mechanism of septic vascular dysfunction. *Crit Care, 11*(5), R107. doi: 10.1186/cc6133

Gerber, H. P., McMurtrey, A., Kowalski, J., Yan, M., Keyt, B. A., Dixit, V., & Ferrara, N. (1998). Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J Biol Chem, 273*(46), 30336-30343.

Gerhardt, H., Golding, M., Fruttiger, M., Ruhrberg, C., Lundkvist, A., Abramsson, A., . . . Betsholtz, C. (2003). VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol, 161*(6), 1163-1177. doi: 10.1083/jcb.200302047

Gottlob, K., Majewski, N., Kennedy, S., Kandel, E., Robey, R. B., & Hay, N. (2001). Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase. *Genes Dev, 15*(11), 1406-1418. doi: 10.1101/gad.889901

Gridley, T. (2007). Notch signaling in vascular development and physiology. *Development, 134*(15), 2709-2718. doi: 10.1242/dev.004184

Gridley, T. (2010). Notch signaling in the vasculature. *Curr Top Dev Biol, 92*, 277-309. doi: 10.1016/S0070-2153(10)92009-7

Gurtner, G. C., Werner, S., Barrandon, Y., & Longaker, M. T. (2008). Wound repair and regeneration. *Nature, 453*(7193), 314-321. doi: 10.1038/nature07039

Gustafsson, A. B., & Gottlieb, R. A. (2007). Bcl-2 family members and apoptosis, taken to heart. *Am J Physiol Cell Physiol, 292*(1), C45-51. doi: 10.1152/ajpcell.00229.2006

Hanahan, D. (1997). Signaling vascular morphogenesis and maintenance. *Science, 277*(5322), 48-50.

Hanahan, D., & Folkman, J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell, 86*(3), 353-364.

Hannan, K. M., & Little, P. J. (1998). Mechanisms regulating the vascular smooth muscle Na/H exchanger (NHE-1) in diabetes. *Biochem Cell Biol, 76*(5), 751-759.

Hellstrom, M., Phng, L. K., Hofmann, J. J., Wallgard, E., Coultas, L., Lindblom, P., . . . Betsholtz, C. (2007). Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature, 445*(7129), 776-780. doi: 10.1038/nature05571

Hill, J. M., Zalos, G., Halcox, J. P., Schenke, W. H., Waclawiw, M. A., Quyyumi, A. A., & Finkel, T. (2003). Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med, 348*(7), 593-600. doi: 10.1056/NEJMoa022287

Hirschi, K. K., Ingram, D. A., & Yoder, M. C. (2008). Assessing identity, phenotype, and fate of endothelial progenitor cells. *Arterioscler Thromb Vasc Biol, 28*(9), 1584-1595. doi: 10.1161/ATVBAHA.107.155960

Hisano, N., Yatomi, Y., Satoh, K., Akimoto, S., Mitsumata, M., Fujino, M. A., & Ozaki, Y. (1999). Induction and suppression of endothelial cell apoptosis by sphingolipids: a possible in vitro model for cell-cell interactions between platelets and endothelial cells. *Blood, 93*(12), 4293-4299.

Hofmann, J. J., & Iruela-Arispe, M. L. (2007). Notch signaling in blood vessels: who is talking to whom about what? *Circ Res, 100*(11), 1556-1568. doi: 10.1161/01.RES.0000266408.42939.e4

Holderfield, M. T., & Hughes, C. C. (2008). Crosstalk between vascular endothelial growth factor, notch, and transforming growth factor-beta in vascular morphogenesis. *Circ Res, 102*(6), 637-652. doi: 10.1161/CIRCRESAHA.107.167171

Hrabe de Angelis, M., McIntyre, J., 2nd, & Gossler, A. (1997). Maintenance of somite borders in mice requires the Delta homologue Dll1. *Nature, 386*(6626), 717-721. doi: 10.1038/386717a0

Huang, L., Critser, P. J., Grimes, B. R., & Yoder, M. C. (2011). Human umbilical cord blood plasma can replace fetal bovine serum for in vitro expansion of functional human endothelial colony-forming cells. *Cytotherapy, 13*(6), 712-721. doi: 10.3109/14653249.2010.548380

Huang, X. T., Zhang, Y. Q., Li, S. J., Li, S. H., Tang, Q., Wang, Z. T., . . . Zhang, J. N. (2013). Intracerebroventricular transplantation of ex vivo expanded endothelial colony-forming cells restores blood-brain barrier integrity and promotes angiogenesis of mice with traumatic brain injury. *J Neurotrauma, 30*(24), 2080-2088. doi: 10.1089/neu.2013.2996

Huppert, S. S., Le, A., Schroeter, E. H., Mumm, J. S., Saxena, M. T., Milner, L. A., & Kopan, R. (2000). Embryonic lethality in mice homozygous for a

processing-deficient allele of Notch1. *Nature, 405*(6789), 966-970. doi: 10.1038/35016111

Hynes, R. O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. *Cell, 69*(1), 11-25.

Ilan, N., Mahooti, S., & Madri, J. A. (1998). Distinct signal transduction pathways are utilized during the tube formation and survival phases of in vitro angiogenesis. *J Cell Sci, 111 (Pt 24)*, 3621-3631.

Ingram, D. A., Mead, L. E., Moore, D. B., Woodard, W., Fenoglio, A., & Yoder, M. C. (2005). Vessel wall-derived endothelial cells rapidly proliferate because they contain a complete hierarchy of endothelial progenitor cells. *Blood, 105*(7), 2783-2786. doi: 10.1182/blood-2004-08-3057

Ingram, D. A., Mead, L. E., Tanaka, H., Meade, V., Fenoglio, A., Mortell, K., . . . Yoder, M. C. (2004). Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. *Blood, 104*(9), 2752-2760. doi: 10.1182/blood-2004-04-1396

Jacobson, M. D., Weil, M., & Raff, M. C. (1997). Programmed cell death in animal development. *Cell, 88*(3), 347-354.

Janiszewski, M., Do Carmo, A. O., Pedro, M. A., Silva, E., Knobel, E., & Laurindo, F. R. (2004). Platelet-derived exosomes of septic individuals possess proapoptotic NAD(P)H oxidase activity: A novel vascular redox pathway. *Crit Care Med, 32*(3), 818-825.

Jehn, B. M., Bielke, W., Pear, W. S., & Osborne, B. A. (1999). Cutting edge: protective effects of notch-1 on TCR-induced apoptosis. *J Immunol, 162*(2), 635-638.

Jeltsch, M., Kaipainen, A., Joukov, V., Meng, X., Lakso, M., Rauvala, H., . . . Alitalo, K. (1997). Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. *Science, 276*(5317), 1423-1425.

Jin, S. W., Beis, D., Mitchell, T., Chen, J. N., & Stainier, D. Y. (2005). Cellular and molecular analyses of vascular tube and lumen formation in zebrafish. *Development, 132*(23), 5199-5209. doi: 10.1242/dev.02087

Jones, R. G., Parsons, M., Bonnard, M., Chan, V. S., Yeh, W. C., Woodgett, J. R., & Ohashi, P. S. (2000). Protein kinase B regulates T lymphocyte survival, nuclear factor kappaB activation, and Bcl-X(L) levels in vivo. *J Exp Med, 191*(10), 1721-1734.

Joutel, A., Andreux, F., Gaulis, S., Domenga, V., Cecillon, M., Battail, N., . . . Tournier-Lasserve, E. (2000). The ectodomain of the Notch3 receptor accumulates within the cerebrovasculature of CADASIL patients. *J Clin Invest, 105*(5), 597-605. doi: 10.1172/JCI8047

Kanda, S., Miyata, Y., & Kanetake, H. (2004). Fibroblast growth factor-2-mediated capillary morphogenesis of endothelial cells requires signals via Flt-1/vascular endothelial growth factor receptor-1: possible involvement of c-Akt. *J Biol Chem, 279*(6), 4007-4016. doi: 10.1074/jbc.M307569200

Kang, K. T., Allen, P., & Bischoff, J. (2011). Bioengineered human vascular networks transplanted into secondary mice reconnect with the host vasculature and re-establish perfusion. *Blood, 118*(25), 6718-6721. doi: 10.1182/blood-2011-08-375188

Kang, K. T., Coggins, M., Xiao, C., Rosenzweig, A., & Bischoff, J. (2013). Human vasculogenic cells form functional blood vessels and mitigate adverse remodeling after ischemia reperfusion injury in rats. *Angiogenesis, 16*(4), 773-784. doi: 10.1007/s10456-013-9354-9

Kanzawa, S., Endo, H., & Shioya, N. (1993). Improved in vitro angiogenesis model by collagen density reduction and the use of type III collagen. *Ann Plast Surg, 30*(3), 244-251.

Kazemi, S., Wenzel, D., Kolossov, E., Lenka, N., Raible, A., Sasse, P., . . . Bloch, W. (2002). Differential role of bFGF and VEGF for vasculogenesis. *Cell Physiol Biochem, 12*(2-3), 55-62. doi: 63781

Kim, H., Huang, L., Critser, P. J., Yang, Z., Chan, R. J., Wang, L., . . . Yoder, M. C. (2015). Notch ligand Delta-like 1 promotes in vivo vasculogenesis in human cord blood-derived endothelial colony forming cells. *Cytotherapy, 17*(5), 579-592. doi: 10.1016/j.jcyt.2014.12.003

Kim, H., Prasain, N., Vemula, S., Ferkowicz, M. J., Yoshimoto, M., Voytik-Harbin, S. L., & Yoder, M. C. (2015). Human platelet lysate improves human cord blood derived ECFC survival and vasculogenesis in three dimensional (3D) collagen matrices. *Microvasc Res, 101*, 72-81. doi: 10.1016/j.mvr.2015.06.006

Kim, I., Kim, H. G., So, J. N., Kim, J. H., Kwak, H. J., & Koh, G. Y. (2000). Angiopoietin-1 regulates endothelial cell survival through the phosphatidylinositol 3'-Kinase/Akt signal transduction pathway. *Circ Res, 86*(1), 24-29.

Kim, Y. H., Hu, H., Guevara-Gallardo, S., Lam, M. T., Fong, S. Y., & Wang, R. A. (2008). Artery and vein size is balanced by Notch and ephrin B2/EphB4 during angiogenesis. *Development, 135*(22), 3755-3764. doi: 135/22/3755 [pii]10.1242/dev.022475

Koh, W., Mahan, R. D., & Davis, G. E. (2008). Cdc42- and Rac1-mediated endothelial lumen formation requires Pak2, Pak4 and Par3, and PKC-dependent signaling. *J Cell Sci, 121*(Pt 7), 989-1001. doi: 10.1242/jcs.020693

Koh, W., Stratman, A. N., Sacharidou, A., & Davis, G. E. (2008). In vitro three dimensional collagen matrix models of endothelial lumen formation during vasculogenesis and angiogenesis. *Methods Enzymol, 443*, 83-101. doi: 10.1016/S0076-6879(08)02005-3

Kontos, C. D., Stauffer, T. P., Yang, W. P., York, J. D., Huang, L., Blanar, M. A., . . . Peters, K. G. (1998). Tyrosine 1101 of Tie2 is the major site of association of p85 and is required for activation of phosphatidylinositol 3-kinase and Akt. *Mol Cell Biol, 18*(7), 4131-4140.

Korff, T., & Augustin, H. G. (1999). Tensional forces in fibrillar extracellular matrices control directional capillary sprouting. *J Cell Sci, 112 (Pt 19)*, 3249-3258.

Kotani, K., Ogawa, W., Hino, Y., Kitamura, T., Ueno, H., Sano, W., . . . Kasuga, M. (1999). Dominant negative forms of Akt (protein kinase B) and atypical protein kinase Clambda do not prevent insulin inhibition of

phosphoenolpyruvate carboxykinase gene transcription. *J Biol Chem, 274*(30), 21305-21312.

Krebs, L. T., Starling, C., Chervonsky, A. V., & Gridley, T. (2010). Notch1 activation in mice causes arteriovenous malformations phenocopied by ephrinB2 and EphB4 mutants. *Genesis, 48*(3), 146-150. doi: 10.1002/dvg.20599

Krebs, L. T., Xue, Y., Norton, C. R., Shutter, J. R., Maguire, M., Sundberg, J. P., . . . Gridley, T. (2000). Notch signaling is essential for vascular morphogenesis in mice. *Genes Dev, 14*(11), 1343-1352.

Kume, T. (2009). Novel insights into the differential functions of Notch ligands in vascular formation. *J Angiogenes Res, 1*, 8. doi: 10.1186/2040-2384-1-8

Kurz, H., Gartner, T., Eggli, P. S., & Christ, B. (1996). First blood vessels in the avian neural tube are formed by a combination of dorsal angioblast immigration and ventral sprouting of endothelial cells. *Dev Biol, 173*(1), 133-147. doi: 10.1006/dbio.1996.0012

LaBonne, C., & Whitman, M. (1994). Mesoderm induction by activin requires FGF-mediated intracellular signals. *Development, 120*(2), 463-472.

Lamalice, L., Le Boeuf, F., & Huot, J. (2007). Endothelial cell migration during angiogenesis. *Circ Res, 100*(6), 782-794. doi: 10.1161/01.RES.0000259593.07661.1e

Langer, H. F., Stellos, K., Steingen, C., Froihofer, A., Schonberger, T., Kramer, B., . . . Gawaz, M. (2009). Platelet derived bFGF mediates vascular integrative mechanisms of mesenchymal stem cells in vitro. *J Mol Cell Cardiol, 47*(2), 315-325. doi: 10.1016/j.yjmcc.2009.03.011

Lanner, F., Sohl, M., & Farnebo, F. (2007). Functional arterial and venous fate is determined by graded VEGF signaling and notch status during embryonic stem cell differentiation. *Arterioscler Thromb Vasc Biol, 27*(3), 487-493. doi: 01.ATV.0000255990.91805.6d [pii] 10.1161/01.ATV.0000255990.91805.6d

Lawson, N. D., Scheer, N., Pham, V. N., Kim, C. H., Chitnis, A. B., Campos-Ortega, J. A., & Weinstein, B. M. (2001). Notch signaling is required for arterial-venous differentiation during embryonic vascular development. *Development, 128*(19), 3675-3683.

Lawson, N. D., Vogel, A. M., & Weinstein, B. M. (2002). sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation. *Dev Cell, 3*(1), 127-136. doi: S1534580702001983 [pii]

Lee, M. Y., Luciano, A. K., Ackah, E., Rodriguez-Vita, J., Bancroft, T. A., Eichmann, A., . . . Sessa, W. C. (2014). Endothelial Akt1 mediates angiogenesis by phosphorylating multiple angiogenic substrates. *Proc Natl Acad Sci U S A, 111*(35), 12865-12870. doi: 10.1073/pnas.1408472111

Lee, S. H., Schloss, D. J., & Swain, J. L. (2000). Maintenance of vascular integrity in the embryo requires signaling through the fibroblast growth factor receptor. *J Biol Chem, 275*(43), 33679-33687. doi: 10.1074/jbc.M004994200

Leslie, J. D., Ariza-McNaughton, L., Bermange, A. L., McAdow, R., Johnson, S. L., & Lewis, J. (2007). Endothelial signalling by the Notch ligand Delta-like 4 restricts angiogenesis. *Development, 134*(5), 839-844. doi: 10.1242/dev.003244

Lev, E. I., Estrov, Z., Aboulfatova, K., Harris, D., Granada, J. F., Alviar, C., . . . Dong, J. F. (2006). Potential role of activated platelets in homing of human endothelial progenitor cells to subendothelial matrix. *Thromb Haemost, 96*(4), 498-504.

Limbourg, A., Ploom, M., Elligsen, D., Sorensen, I., Ziegelhoeffer, T., Gossler, A., . . . Limbourg, F. P. (2007). Notch ligand Delta-like 1 is essential for postnatal arteriogenesis. *Circ Res, 100*(3), 363-371. doi: 10.1161/01.RES.0000258174.77370.2c

Lin, R. Z., Moreno-Luna, R., Zhou, B., Pu, W. T., & Melero-Martin, J. M. (2012). Equal modulation of endothelial cell function by four distinct tissue-specific mesenchymal stem cells. *Angiogenesis, 15*(3), 443-455. doi: 10.1007/s10456-012-9272-2

Liu, Y., & Senger, D. R. (2004). Matrix-specific activation of Src and Rho initiates capillary morphogenesis of endothelial cells. *FASEB J, 18*(3), 457-468. doi: 10.1096/fj.03-0948com

Liu, Z. J., Shirakawa, T., Li, Y., Soma, A., Oka, M., Dotto, G. P., . . . Herlyn, M. (2003). Regulation of Notch1 and Dll4 by vascular endothelial growth factor in arterial endothelial cells: implications for modulating arteriogenesis and angiogenesis. *Mol Cell Biol, 23*(1), 14-25.

Llevadot, J., & Asahara, T. (2002). [Effects of statins on angiogenesis and vasculogenesis]. *Rev Esp Cardiol, 55*(8), 838-844.

Lobov, I. B., Renard, R. A., Papadopoulos, N., Gale, N. W., Thurston, G., Yancopoulos, G. D., & Wiegand, S. J. (2007). Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. *Proc Natl Acad Sci U S A, 104*(9), 3219-3224. doi: 10.1073/pnas.0611206104

Luo, Y., Shoemaker, A. R., Liu, X., Woods, K. W., Thomas, S. A., de Jong, R., . . . Giranda, V. L. (2005). Potent and selective inhibitors of Akt kinases slow the progress of tumors in vivo. *Mol Cancer Ther, 4*(6), 977-986. doi: 10.1158/1535-7163.MCT-05-0005

MacKenzie, F., Duriez, P., Wong, F., Noseda, M., & Karsan, A. (2004). Notch4 inhibits endothelial apoptosis via RBP-Jkappa-dependent and -independent pathways. *J Biol Chem, 279*(12), 11657-11663. doi: 10.1074/jbc.M312102200

Magnusson, P., Rolny, C., Jakobsson, L., Wikner, C., Wu, Y., Hicklin, D. J., & Claesson-Welsh, L. (2004). Deregulation of Flk-1/vascular endothelial growth factor receptor-2 in fibroblast growth factor receptor-1-deficient vascular stem cell development. *J Cell Sci, 117*(Pt 8), 1513-1523. doi: 10.1242/jcs.00999

Matsumura, T., Wolff, K., & Petzelbauer, P. (1997). Endothelial cell tube formation depends on cadherin 5 and CD31 interactions with filamentous actin. *J Immunol, 158*(7), 3408-3416.

Mause, S. F., Ritzel, E., Liehn, E. A., Hristov, M., Bidzhekov, K., Muller-Newen, G., . . . Weber, C. (2010). Platelet microparticles enhance the vasoregenerative potential of angiogenic early outgrowth cells after vascular injury. *Circulation, 122*(5), 495-506. doi: 10.1161/CIRCULATIONAHA.109.909473

Mause, S. F., von Hundelshausen, P., Zernecke, A., Koenen, R. R., & Weber, C. (2005). Platelet microparticles: a transcellular delivery system for RANTES promoting monocyte recruitment on endothelium. *Arterioscler Thromb Vasc Biol, 25*(7), 1512-1518. doi: 10.1161/01.ATV.0000170133.43608.37

Medina, R. J., O'Neill, C. L., Humphreys, M. W., Gardiner, T. A., & Stitt, A. W. (2010). Outgrowth endothelial cells: characterization and their potential for reversing ischemic retinopathy. *Invest Ophthalmol Vis Sci, 51*(11), 5906-5913. doi: 10.1167/iovs.09-4951

Melero-Martin, J. M., De Obaldia, M. E., Allen, P., Dudley, A. C., Klagsbrun, M., & Bischoff, J. (2010). Host myeloid cells are necessary for creating bioengineered human vascular networks in vivo. *Tissue Eng Part A, 16*(8), 2457-2466. doi: 10.1089/ten.TEA.2010.0024

Melero-Martin, J. M., De Obaldia, M. E., Kang, S. Y., Khan, Z. A., Yuan, L., Oettgen, P., & Bischoff, J. (2008). Engineering robust and functional vascular networks in vivo with human adult and cord blood-derived progenitor cells. *Circ Res, 103*(2), 194-202. doi: 10.1161/CIRCRESAHA.108.178590 CIRCRESAHA.108.178590 [pii]

Melero-Martin, J. M., Khan, Z. A., Picard, A., Wu, X., Paruchuri, S., & Bischoff, J. (2007). In vivo vasculogenic potential of human blood-derived endothelial progenitor cells. *Blood, 109*(11), 4761-4768. doi: 10.1182/blood-2006-12-062471

Ment, L. R., Stewart, W. B., Scaramuzzino, D., & Madri, J. A. (1997). An in vitro three-dimensional coculture model of cerebral microvascular angiogenesis and differentiation. *In Vitro Cell Dev Biol Anim, 33*(9), 684-691. doi: 10.1007/s11626-997-0126-y

Millauer, B., Shawver, L. K., Plate, K. H., Risau, W., & Ullrich, A. (1994). Glioblastoma growth inhibited in vivo by a dominant-negative Flk-1 mutant. *Nature, 367*(6463), 576-579. doi: 10.1038/367576a0

Millauer, B., Wizigmann-Voos, S., Schnurch, H., Martinez, R., Moller, N. P., Risau, W., & Ullrich, A. (1993). High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell, 72*(6), 835-846.

Miller, D. L., Ortega, S., Bashayan, O., Basch, R., & Basilico, C. (2000). Compensation by fibroblast growth factor 1 (FGF1) does not account for the mild phenotypic defects observed in FGF2 null mice. *Mol Cell Biol, 20*(6), 2260-2268.

Morales-Ruiz, M., Fulton, D., Sowa, G., Languino, L. R., Fujio, Y., Walsh, K., & Sessa, W. C. (2000). Vascular endothelial growth factor-stimulated actin reorganization and migration of endothelial cells is regulated via the serine/threonine kinase Akt. *Circ Res, 86*(8), 892-896.

Moubarik, C., Guillet, B., Youssef, B., Codaccioni, J. L., Piercecchi, M. D., Sabatier, F., . . . Pisano, P. (2011). Transplanted late outgrowth endothelial progenitor cells as cell therapy product for stroke. *Stem Cell Rev, 7*(1), 208-220. doi: 10.1007/s12015-010-9157-y

Mund, J. A., Estes, M. L., Yoder, M. C., Ingram, D. A., Jr., & Case, J. (2012). Flow cytometric identification and functional characterization of immature and mature circulating endothelial cells. *Arterioscler Thromb Vasc Biol, 32*(4), 1045-1053. doi: 10.1161/ATVBAHA.111.244210

Nakamura, T., Teramoto, H., & Ichihara, A. (1986). Purification and characterization of a growth factor from rat platelets for mature parenchymal hepatocytes in primary cultures. *Proc Natl Acad Sci U S A, 83*(17), 6489-6493.

Neufeld, G., Cohen, T., Gengrinovitch, S., & Poltorak, Z. (1999). Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J, 13*(1), 9-22.

Ng, C. P., Helm, C. L., & Swartz, M. A. (2004). Interstitial flow differentially stimulates blood and lymphatic endothelial cell morphogenesis in vitro. *Microvasc Res, 68*(3), 258-264. doi: 10.1016/j.mvr.2004.08.002

Niessen, K., & Karsan, A. (2008). Notch signaling in cardiac development. *Circ Res, 102*(10), 1169-1181. doi: 102/10/1169 [pii] 10.1161/CIRCRESAHA.108.174318

Noguera-Troise, I., Daly, C., Papadopoulos, N. J., Coetzee, S., Boland, P., Gale, N. W., . . . Thurston, G. (2006). Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. *Nature, 444*(7122), 1032-1037. doi: 10.1038/nature05355

Ohishi, K., Varnum-Finney, B., Flowers, D., Anasetti, C., Myerson, D., & Bernstein, I. D. (2000). Monocytes express high amounts of Notch and undergo cytokine specific apoptosis following interaction with the Notch ligand, Delta-1. *Blood, 95*(9), 2847-2854.

Olofsson, B., Pajusola, K., Kaipainen, A., von Euler, G., Joukov, V., Saksela, O., . . . Eriksson, U. (1996). Vascular endothelial growth factor B, a novel growth factor for endothelial cells. *Proc Natl Acad Sci U S A, 93*(6), 2576-2581.

Ornitz, D. M., & Itoh, N. (2001). Fibroblast growth factors. *Genome Biol, 2*(3), REVIEWS3005.

Pajusola, K., Aprelikova, O., Pelicci, G., Weich, H., Claesson-Welsh, L., & Alitalo, K. (1994). Signalling properties of FLT4, a proteolytically processed receptor tyrosine kinase related to two VEGF receptors. *Oncogene, 9*(12), 3545-3555.

Pakala, R., Willerson, J. T., & Benedict, C. R. (1994). Mitogenic effect of serotonin on vascular endothelial cells. *Circulation, 90*(4), 1919-1926.

Papapetropoulos, A., Fulton, D., Mahboubi, K., Kalb, R. G., O'Connor, D. S., Li, F., . . . Sessa, W. C. (2000). Angiopoietin-1 inhibits endothelial cell apoptosis via the Akt/survivin pathway. *J Biol Chem, 275*(13), 9102-9105.

Paradis, S., & Ruvkun, G. (1998). Caenorhabditis elegans Akt/PKB transduces insulin receptor-like signals from AGE-1 PI3 kinase to the DAF-16 transcription factor. *Genes Dev, 12*(16), 2488-2498.

Peng, X. D., Xu, P. Z., Chen, M. L., Hahn-Windgassen, A., Skeen, J., Jacobs, J., . . . Hay, N. (2003). Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2. *Genes Dev, 17*(11), 1352-1365. doi: 10.1101/gad.1089403

Petrova, T. V., Makinen, T., & Alitalo, K. (1999). Signaling via vascular endothelial growth factor receptors. *Exp Cell Res, 253*(1), 117-130. doi: 10.1006/excr.1999.4707

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res, 29*(9), e45.

Phng, L. K., & Gerhardt, H. (2009). Angiogenesis: a team effort coordinated by notch. *Dev Cell, 16*(2), 196-208. doi: 10.1016/j.devcel.2009.01.015

Plas, D. R., Talapatra, S., Edinger, A. L., Rathmell, J. C., & Thompson, C. B. (2001). Akt and Bcl-xL promote growth factor-independent survival through distinct effects on mitochondrial physiology. *J Biol Chem, 276*(15), 12041-12048. doi: 10.1074/jbc.M010551200

Pollman, M. J., Naumovski, L., & Gibbons, G. H. (1999). Endothelial cell apoptosis in capillary network remodeling. *J Cell Physiol, 178*(3), 359-370. doi: 10.1002/(SICI)1097-4652(199903)178:3<359::AID-JCP10>3.0.CO;2-O

Prindull, G. (1995). Apoptosis in the embryo and tumorigenesis. *Eur J Cancer, 31A*(1), 116-123.

Radtke, F., Fasnacht, N., & Macdonald, H. R. (2010). Notch signaling in the immune system. *Immunity, 32*(1), 14-27. doi: S1074-7613(10)00005-1 [pii] 10.1016/j.immuni.2010.01.004

Rafii, S., & Lyden, D. (2003). Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. *Nat Med, 9*(6), 702-712. doi: 10.1038/nm0603-702

Rafii, S., Lyden, D., Benezra, R., Hattori, K., & Heissig, B. (2002). Vascular and haematopoietic stem cells: novel targets for anti-angiogenesis therapy? *Nat Rev Cancer, 2*(11), 826-835. doi: 10.1038/nrc925

Ranta, V., Mikkola, T., Ylikorkala, O., Viinikka, L., & Orpana, A. (1998). Reduced viability of human vascular endothelial cells cultured on Matrigel. *J Cell Physiol, 176*(1), 92-98. doi: 10.1002/(SICI)1097-4652(199807)176:1<92::AID-JCP11>3.0.CO;2-Q

Reinisch, A., Hofmann, N. A., Obenauf, A. C., Kashofer, K., Rohde, E., Schallmoser, K., . . . Strunk, D. (2009). Humanized large-scale expanded endothelial colony-forming cells function in vitro and in vivo. *Blood, 113*(26), 6716-6725. doi: 10.1182/blood-2008-09-181362

Ridgway, J., Zhang, G., Wu, Y., Stawicki, S., Liang, W. C., Chanthery, Y., . . . Yan, M. (2006). Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. *Nature, 444*(7122), 1083-1087. doi: 10.1038/nature05313

Risau, W. (1995). Differentiation of endothelium. *FASEB J, 9*(10), 926-933.

Risau, W. (1997). Mechanisms of angiogenesis. *Nature, 386*(6626), 671-674. doi: 10.1038/386671a0

Risau, W., & Flamme, I. (1995). Vasculogenesis. *Annu Rev Cell Dev Biol, 11*, 73-91. doi: 10.1146/annurev.cb.11.110195.000445

Roca, C., & Adams, R. H. (2007). Regulation of vascular morphogenesis by Notch signaling. *Genes Dev, 21*(20), 2511-2524. doi: 10.1101/gad.1589207

Rocha, S. F., & Adams, R. H. (2009). Molecular differentiation and specialization of vascular beds. *Angiogenesis, 12*(2), 139-147. doi: 10.1007/s10456-009-9132-x

Rohban, R., Reinisch, A., Etchart, N., Schallmoser, K., Hofmann, N. A., Szoke, K., . . . Strunk, D. (2013). Identification of an effective early signaling signature during neo-vasculogenesis in vivo by ex vivo proteomic profiling. *PLoS One, 8*(6), e66909. doi: 10.1371/journal.pone.0066909

Roman, B. L., & Weinstein, B. M. (2000). Building the vertebrate vasculature: research is going swimmingly. *Bioessays, 22*(10), 882-893. doi: 10.1002/1521-1878(200010)22:10<882::AID-BIES3>3.0.CO;2-J

Rossant, J., & Howard, L. (2002). Signaling pathways in vascular development. *Annu Rev Cell Dev Biol, 18*, 541-573. doi: 10.1146/annurev.cellbio.18.012502.105825012502.105825 [pii]

Rozmyslowicz, T., Majka, M., Kijowski, J., Murphy, S. L., Conover, D. O., Poncz, M., . . . Ratajczak, M. Z. (2003). Platelet- and megakaryocyte-derived microparticles transfer CXCR4 receptor to CXCR4-null cells and make them susceptible to infection by X4-HIV. *AIDS, 17*(1), 33-42. doi: 10.1097/01.aids.0000042948.95433.3d

Sacharidou, A., Stratman, A. N., & Davis, G. E. (2012). Molecular mechanisms controlling vascular lumen formation in three-dimensional extracellular matrices. *Cells Tissues Organs, 195*(1-2), 122-143. doi: 10.1159/000331410

Sade, H., Krishna, S., & Sarin, A. (2004). The anti-apoptotic effect of Notch-1 requires p56lck-dependent, Akt/PKB-mediated signaling in T cells. *J Biol Chem, 279*(4), 2937-2944. doi: 10.1074/jbc.M309924200

Sainson, R. C., Aoto, J., Nakatsu, M. N., Holderfield, M., Conn, E., Koller, E., & Hughes, C. C. (2005). Cell-autonomous notch signaling regulates endothelial cell branching and proliferation during vascular tubulogenesis. *FASEB J, 19*(8), 1027-1029. doi: 10.1096/fj.04-3172fje

Salazar, R., Bell, S. E., & Davis, G. E. (1999). Coordinate induction of the actin cytoskeletal regulatory proteins gelsolin, vasodilator-stimulated phosphoprotein, and profilin during capillary morphogenesis in vitro. *Exp Cell Res, 249*(1), 22-32. doi: 10.1006/excr.1999.4460

Savill, J., & Fadok, V. (2000). Corpse clearance defines the meaning of cell death. *Nature, 407*(6805), 784-788. doi: 10.1038/35037722

Schallmoser, K., & Strunk, D. (2009). Preparation of pooled human platelet lysate (pHPL) as an efficient supplement for animal serum-free human stem cell cultures. *J Vis Exp*(32). doi: 10.3791/1523

Schechner, J. S., Nath, A. K., Zheng, L., Kluger, M. S., Hughes, C. C., Sierra-Honigmann, M. R., . . . Pober, J. S. (2000). In vivo formation of complex microvessels lined by human endothelial cells in an immunodeficient

mouse. *Proc Natl Acad Sci U S A, 97*(16), 9191-9196. doi: 10.1073/pnas.150242297

Schmitt, T. M., & Zuniga-Pflucker, J. C. (2002). Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. *Immunity, 17*(6), 749-756. doi: S1074761302004740 [pii]

Schwarz, T. M., Leicht, S. F., Radic, T., Rodriguez-Araboalaza, I., Hermann, P. C., Berger, F., . . . Heeschen, C. (2012). Vascular incorporation of endothelial colony-forming cells is essential for functional recovery of murine ischemic tissue following cell therapy. *Arterioscler Thromb Vasc Biol, 32*(2), e13-21. doi: 10.1161/ATVBAHA.111.239822

Scorrano, L., & Korsmeyer, S. J. (2003). Mechanisms of cytochrome c release by proapoptotic BCL-2 family members. *Biochem Biophys Res Commun, 304*(3), 437-444.

Seandel, M., Butler, J. M., Kobayashi, H., Hooper, A. T., White, I. A., Zhang, F., . . . Rafii, S. (2008). Generation of a functional and durable vascular niche by the adenoviral E4ORF1 gene. *Proc Natl Acad Sci U S A, 105*(49), 19288-19293. doi: 10.1073/pnas.0805980105

Seghezzi, G., Patel, S., Ren, C. J., Gualandris, A., Pintucci, G., Robbins, E. S., . . . Mignatti, P. (1998). Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: an autocrine mechanism contributing to angiogenesis. *J Cell Biol, 141*(7), 1659-1673.

Segura, I., Serrano, A., De Buitrago, G. G., Gonzalez, M. A., Abad, J. L., Claveria, C., . . . Riese, H. H. (2002). Inhibition of programmed cell death impairs in vitro vascular-like structure formation and reduces in vivo angiogenesis. *FASEB J, 16*(8), 833-841. doi: 10.1096/fj.01-0819com

Shalaby, F., Ho, J., Stanford, W. L., Fischer, K. D., Schuh, A. C., Schwartz, L., . . . Rossant, J. (1997). A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. *Cell, 89*(6), 981-990.

Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X. F., Breitman, M. L., & Schuh, A. C. (1995). Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature, 376*(6535), 62-66. doi: 10.1038/376062a0

Sharron, M., Hoptay, C. E., Wiles, A. A., Garvin, L. M., Geha, M., Benton, A. S., . . . Freishtat, R. J. (2012). Platelets induce apoptosis during sepsis in a contact-dependent manner that is inhibited by GPIIb/IIIa blockade. *PLoS One, 7*(7), e41549. doi: 10.1371/journal.pone.0041549

Shawber, C. J., & Kitajewski, J. (2004). Notch function in the vasculature: insights from zebrafish, mouse and man. *Bioessays, 26*(3), 225-234. doi: 10.1002/bies.20004

Shelley, W. C., Leapley, A. C., Huang, L., Critser, P. J., Zeng, P., Prater, D., . . . Yoder, M. C. (2012). Changes in the frequency and in vivo vessel-forming ability of rhesus monkey circulating endothelial colony-forming cells across the lifespan (birth to aged). *Pediatr Res, 71*(2), 156-161. doi: 10.1038/pr.2011.22

Shepherd, B. R., Enis, D. R., Wang, F., Suarez, Y., Pober, J. S., & Schechner, J. S. (2006). Vascularization and engraftment of a human skin substitute using circulating progenitor cell-derived endothelial cells. *The FASEB Journal, 20*(10), 1739-1741. doi: 10.1096/fj.05-5682fje

Shepherd, B. R., Jay, S. M., Saltzman, W. M., Tellides, G., & Pober, J. S. (2009). Human aortic smooth muscle cells promote arteriole formation by coengrafted endothelial cells. *Tissue Eng Part A, 15*(1), 165-173. doi: 10.1089/ten.tea.2008.0010

Shi, Q., Rafii, S., Wu, M. H., Wijelath, E. S., Yu, C., Ishida, A., . . . Hammond, W. P. (1998). Evidence for circulating bone marrow-derived endothelial cells. *Blood, 92*(2), 362-367.

Shih, D. T., Chen, J. C., Chen, W. Y., Kuo, Y. P., Su, C. Y., & Burnouf, T. (2011). Expansion of adipose tissue mesenchymal stromal progenitors in serum-free medium supplemented with virally inactivated allogeneic human platelet lysate. *Transfusion, 51*(4), 770-778. doi: 10.1111/j.1537-2995.2010.02915.x

Shoemaker, A. R., Mitten, M. J., Adickes, J., Ackler, S., Refici, M., Ferguson, D., . . . Elmore, S. W. (2008). Activity of the Bcl-2 family inhibitor ABT-263 in a panel of small cell lung cancer xenograft models. *Clin Cancer Res, 14*(11), 3268-3277. doi: 10.1158/1078-0432.CCR-07-4622

Siekmann, A. F., Covassin, L., & Lawson, N. D. (2008). Modulation of VEGF signalling output by the Notch pathway. *Bioessays, 30*(4), 303-313. doi: 10.1002/bies.20736

Siekmann, A. F., & Lawson, N. D. (2007). Notch signalling limits angiogenic cell behaviour in developing zebrafish arteries. *Nature, 445*(7129), 781-784. doi: 10.1038/nature05577

Sieminski, A. L., Hebbel, R. P., & Gooch, K. J. (2004). The relative magnitudes of endothelial force generation and matrix stiffness modulate capillary morphogenesis in vitro. *Exp Cell Res, 297*(2), 574-584. doi: 10.1016/j.yexcr.2004.03.035

Singer, A. J., & Clark, R. A. (1999). Cutaneous wound healing. *N Engl J Med, 341*(10), 738-746. doi: 10.1056/NEJM199909023411006

Slack, J. M., Darlington, B. G., Heath, J. K., & Godsave, S. F. (1987). Mesoderm induction in early Xenopus embryos by heparin-binding growth factors. *Nature, 326*(6109), 197-200. doi: 10.1038/326197a0

Sorensen, I., Adams, R. H., & Gossler, A. (2009). DLL1-mediated Notch activation regulates endothelial identity in mouse fetal arteries. *Blood, 113*(22), 5680-5688. doi: blood-2008-08-174508 [pii]10.1182/blood-2008-08-174508

Sprick, M. R., & Walczak, H. (2004). The interplay between the Bcl-2 family and death receptor-mediated apoptosis. *Biochim Biophys Acta, 1644*(2-3), 125-132. doi: 10.1016/j.bbamcr.2003.11.002

Stellos, K., & Gawaz, M. (2007). Platelets and stromal cell-derived factor-1 in progenitor cell recruitment. *Semin Thromb Hemost, 33*(2), 159-164. doi: 10.1055/s-2007-969029

Stratman, A. N., Davis, M. J., & Davis, G. E. (2011). VEGF and FGF prime vascular tube morphogenesis and sprouting directed by hematopoietic stem cell cytokines. *Blood, 117*(14), 3709-3719. doi: 10.1182/blood-2010-11-316752

Stratman, A. N., Saunders, W. B., Sacharidou, A., Koh, W., Fisher, K. E., Zawieja, D. C., . . . Davis, G. E. (2009). Endothelial cell lumen and vascular guidance tunnel formation requires MT1-MMP-dependent proteolysis in 3-dimensional collagen matrices. *Blood, 114*(2), 237-247. doi: 10.1182/blood-2008-12-196451

Stratman, A. N., Schwindt, A. E., Malotte, K. M., & Davis, G. E. (2010). Endothelial-derived PDGF-BB and HB-EGF coordinately regulate pericyte recruitment during vasculogenic tube assembly and stabilization. *Blood, 116*(22), 4720-4730. doi: 10.1182/blood-2010-05-286872

Stromblad, S., Becker, J. C., Yebra, M., Brooks, P. C., & Cheresh, D. A. (1996). Suppression of p53 activity and p21WAF1/CIP1 expression by vascular cell integrin alphaVbeta3 during angiogenesis. *J Clin Invest, 98*(2), 426-433. doi: 10.1172/JCI118808

Stroncek, J. D., Ren, L. C., Klitzman, B., & Reichert, W. M. (2012). Patient-derived endothelial progenitor cells improve vascular graft patency in a rodent model. *Acta Biomater, 8*(1), 201-208. doi: 10.1016/j.actbio.2011.09.002

Stump, M. M., Jordan, G. L., Jr., Debakey, M. E., & Halpert, B. (1963). Endothelium Grown from Circulating Blood on Isolated Intravascular Dacron Hub. *Am J Pathol, 43*, 361-367.

Suchting, S., Freitas, C., le Noble, F., Benedito, R., Breant, C., Duarte, A., & Eichmann, A. (2007). The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. *Proc Natl Acad Sci U S A, 104*(9), 3225-3230. doi: 10.1073/pnas.0611177104

Swift, M. R., & Weinstein, B. M. (2009). Arterial-venous specification during development. *Circ Res, 104*(5), 576-588. doi: 104/5/576 [pii] 10.1161/CIRCRESAHA.108.188805

Takeshita, K., Satoh, M., Ii, M., Silver, M., Limbourg, F. P., Mukai, Y., . . . Liao, J. K. (2007). Critical role of endothelial Notch1 signaling in postnatal angiogenesis. *Circ Res, 100*(1), 70-78. doi: 01.RES.0000254788.47304.6 [pii] 10.1161/01.RES.0000254788.47304.6e

Taylor, R. C., Cullen, S. P., & Martin, S. J. (2008). Apoptosis: controlled demolition at the cellular level. *Nat Rev Mol Cell Biol, 9*(3), 231-241. doi: 10.1038/nrm2312

Tobe, T., Ortega, S., Luna, J. D., Ozaki, H., Okamoto, N., Derevjanik, N. L., . . . Campochiaro, P. A. (1998). Targeted disruption of the FGF2 gene does not prevent choroidal neovascularization in a murine model. *Am J Pathol, 153*(5), 1641-1646. doi: 10.1016/S0002-9440(10)65753-7

Traktuev, D. O., Prater, D. N., Merfeld-Clauss, S., Sanjeevaiah, A. R., Saadatzadeh, M. R., Murphy, M., . . . March, K. L. (2009). Robust functional vascular network formation in vivo by cooperation of adipose

progenitor and endothelial cells. *Circ Res, 104*(12), 1410-1420. doi: 10.1161/CIRCRESAHA.108.190926

Trindade, A., Kumar, S. R., Scehnet, J. S., Lopes-da-Costa, L., Becker, J., Jiang, W., . . . Duarte, A. (2008). Overexpression of delta-like 4 induces arterialization and attenuates vessel formation in developing mouse embryos. *Blood, 112*(5), 1720-1729. doi: blood-2007-09-112748 [pii] 10.1182/blood-2007-09-112748

Tse, C., Shoemaker, A. R., Adickes, J., Anderson, M. G., Chen, J., Jin, S., . . . Elmore, S. W. (2008). ABT-263: a potent and orally bioavailable Bcl-2 family inhibitor. *Cancer Res, 68*(9), 3421-3428. doi: 10.1158/0008-5472.CAN-07-5836

Uchiyama, T., Engelman, R. M., Maulik, N., & Das, D. K. (2004). Role of Akt signaling in mitochondrial survival pathway triggered by hypoxic preconditioning. *Circulation, 109*(24), 3042-3049. doi: 10.1161/01.CIR.0000130647.29030.90

Urbich, C., & Dimmeler, S. (2004). Endothelial progenitor cells: characterization and role in vascular biology. *Circ Res, 95*(4), 343-353. doi: 10.1161/01.RES.0000137877.89448.78

Uyttendaele, H., Ho, J., Rossant, J., & Kitajewski, J. (2001). Vascular patterning defects associated with expression of activated Notch4 in embryonic endothelium. *Proc Natl Acad Sci U S A, 98*(10), 5643-5648. doi: 10.1073/pnas.09158459898/10/5643 [pii]

VanWijk, M. J., VanBavel, E., Sturk, A., & Nieuwland, R. (2003). Microparticles in cardiovascular diseases. *Cardiovasc Res, 59*(2), 277-287.

Varnum-Finney, B., Wu, L., Yu, M., Brashem-Stein, C., Staats, S., Flowers, D., . . . Bernstein, I. D. (2000). Immobilization of Notch ligand, Delta-1, is required for induction of notch signaling. *J Cell Sci, 113 Pt 23*, 4313-4318.

Vasina, E. M., Cauwenberghs, S., Staudt, M., Feijge, M. A., Weber, C., Koenen, R. R., & Heemskerk, J. W. (2013). Aging- and activation-induced platelet microparticles suppress apoptosis in monocytic cells and differentially signal to proinflammatory mediator release. *Am J Blood Res, 3*(2), 107-123.

Vernon, R. B., & Sage, E. H. (1999). A novel, quantitative model for study of endothelial cell migration and sprout formation within three-dimensional collagen matrices. *Microvasc Res, 57*(2), 118-133. doi: 10.1006/mvre.1998.2122

Villa, N., Walker, L., Lindsell, C. E., Gasson, J., Iruela-Arispe, M. L., & Weinmaster, G. (2001). Vascular expression of Notch pathway receptors and ligands is restricted to arterial vessels. *Mech Dev, 108*(1-2), 161-164.

Werner, N., Kosiol, S., Schiegl, T., Ahlers, P., Walenta, K., Link, A., . . . Nickenig, G. (2005). Circulating endothelial progenitor cells and cardiovascular outcomes. *N Engl J Med, 353*(10), 999-1007. doi: 10.1056/NEJMoa043814

Whittington, C. F., Yoder, M. C., & Voytik-Harbin, S. L. (2013). Collagen-polymer guidance of vessel network formation and stabilization by endothelial

colony forming cells in vitro. *Macromol Biosci, 13*(9), 1135-1149. doi: 10.1002/mabi.201300128

Wilting, J., & Christ, B. (1996). Embryonic angiogenesis: a review. *Naturwissenschaften, 83*(4), 153-164.

Woywodt, A., Erdbruegger, U., & Haubitz, M. (2006). Circulating endothelial cells and endothelial progenitor cells after angioplasty: news from the endothelial rescue squad. *J Thromb Haemost, 4*(5), 976-978. doi: 10.1111/j.1538-7836.2006.01920.x

Wu, J., & Bresnick, E. H. (2007). Glucocorticoid and growth factor synergism requirement for Notch4 chromatin domain activation. *Mol Cell Biol, 27*(6), 2411-2422. doi: 10.1128/MCB.02152-06

Wu, J., Iwata, F., Grass, J. A., Osborne, C. S., Elnitski, L., Fraser, P., . . . Bresnick, E. H. (2005). Molecular determinants of NOTCH4 transcription in vascular endothelium. *Mol Cell Biol, 25*(4), 1458-1474. doi: 10.1128/MCB.25.4.1458-1474.2005

Wu, X., Rabkin-Aikawa, E., Guleserian, K. J., Perry, T. E., Masuda, Y., Sutherland, F. W., . . . Bischoff, J. (2004). Tissue-engineered microvessels on three-dimensional biodegradable scaffolds using human endothelial progenitor cells. *Am J Physiol Heart Circ Physiol, 287*(2), H480-487. doi: 10.1152/ajpheart.01232.2003

Wyllie, A. H. (2010). "Where, O death, is thy sting?" A brief review of apoptosis biology. *Mol Neurobiol, 42*(1), 4-9. doi: 10.1007/s12035-010-8125-5

Xu, X., Weinstein, M., Li, C., Naski, M., Cohen, R. I., Ornitz, D. M., . . . Deng, C. (1998). Fibroblast growth factor receptor 2 (FGFR2)-mediated reciprocal regulation loop between FGF8 and FGF10 is essential for limb induction. *Development, 125*(4), 753-765.

Xue, Y., Gao, X., Lindsell, C. E., Norton, C. R., Chang, B., Hicks, C., . . . Gridley, T. (1999). Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. *Hum Mol Genet, 8*(5), 723-730.

Yamaguchi, T. P., Dumont, D. J., Conlon, R. A., Breitman, M. L., & Rossant, J. (1993). flk-1, an flt-related receptor tyrosine kinase is an early marker for endothelial cell precursors. *Development, 118*(2), 489-498.

Yamaguchi, T. P., Harpal, K., Henkemeyer, M., & Rossant, J. (1994). fgfr-1 is required for embryonic growth and mesodermal patterning during mouse gastrulation. *Genes Dev, 8*(24), 3032-3044.

Yang, S., Graham, J., Kahn, J. W., Schwartz, E. A., & Gerritsen, M. E. (1999). Functional roles for PECAM-1 (CD31) and VE-cadherin (CD144) in tube assembly and lumen formation in three-dimensional collagen gels. *Am J Pathol, 155*(3), 887-895. doi: 10.1016/S0002-9440(10)65188-7

Yang, Z., Kondo, T., Voorhorst, C. S., Nabinger, S. C., Ndong, L., Yin, F., . . . Chan, R. J. (2009). Increased c-Jun expression and reduced GATA2 expression promote aberrant monocytic differentiation induced by activating PTPN11 mutants. *Mol Cell Biol, 29*(16), 4376-4393. doi: MCB.01330-08 [pii]10.1128/MCB.01330-08

Yang, Z. Z., Tschopp, O., Di-Poi, N., Bruder, E., Baudry, A., Dummler, B., . . . Hemmings, B. A. (2005). Dosage-dependent effects of Akt1/protein kinase

Balpha (PKBalpha) and Akt3/PKBgamma on thymus, skin, and cardiovascular and nervous system development in mice. *Mol Cell Biol, 25*(23), 10407-10418. doi: 10.1128/MCB.25.23.10407-10418.2005

Yao, R., & Cooper, G. M. (1995). Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science, 267*(5206), 2003-2006.

Yap, T. A., Walton, M. I., Hunter, L. J., Valenti, M., de Haven Brandon, A., Eve, P. D., . . . Garrett, M. D. (2011). Preclinical pharmacology, antitumor activity, and development of pharmacodynamic markers for the novel, potent AKT inhibitor CCT128930. *Mol Cancer Ther, 10*(2), 360-371. doi: 10.1158/1535-7163.MCT-10-0760

Yoder, M. C., Mead, L. E., Prater, D., Krier, T. R., Mroueh, K. N., Li, F., . . . Ingram, D. A. (2007). Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. *Blood, 109*(5), 1801-1809. doi: 10.1182/blood-2006-08-043471

Zernecke, A., Schober, A., Bot, I., von Hundelshausen, P., Liehn, E. A., Mopps, B., . . . Weber, C. (2005). SDF-1alpha/CXCR4 axis is instrumental in neointimal hyperplasia and recruitment of smooth muscle progenitor cells. *Circ Res, 96*(7), 784-791. doi: 10.1161/01.RES.0000162100.52009.38

Zhang, G., Zhou, J., Fan, Q., Zheng, Z., Zhang, F., Liu, X., & Hu, S. (2008). Arterial-venous endothelial cell fate is related to vascular endothelial growth factor and Notch status during human bone mesenchymal stem cell differentiation. *FEBS Lett, 582*(19), 2957-2964. doi: S0014-5793(08)00622-4 [pii]10.1016/j.febslet.2008.07.031

Zheng, L., Dengler, T. J., Kluger, M. S., Madge, L. A., Schechner, J. S., Maher, S. E., . . . Bothwell, A. L. (2000). Cytoprotection of human umbilical vein endothelial cells against apoptosis and CTL-mediated lysis provided by caspase-resistant Bcl-2 without alterations in growth or activation responses. *J Immunol, 164*(9), 4665-4671.

Zhong, T. P., Childs, S., Leu, J. P., & Fishman, M. C. (2001). Gridlock signalling pathway fashions the first embryonic artery. *Nature, 414*(6860), 216-220. doi: 10.1038/3510259935102599 [pii]

Zhou, M., Sutliff, R. L., Paul, R. J., Lorenz, J. N., Hoying, J. B., Haudenschild, C. C., . . . Doetschman, T. (1998). Fibroblast growth factor 2 control of vascular tone. *Nat Med, 4*(2), 201-207.

Zhu, Q. S., Ren, W., Korchin, B., Lahat, G., Dicker, A., Lu, Y., . . . Lev, D. (2008). Soft tissue sarcoma cells are highly sensitive to AKT blockade: a role for p53-independent up-regulation of GADD45 alpha. *Cancer Res, 68*(8), 2895-2903. doi: 10.1158/0008-5472.CAN-07-6268

Zong, W. X., Edelstein, L. C., Chen, C., Bash, J., & Gelinas, C. (1999). The prosurvival Bcl-2 homolog Bfl-1/A1 is a direct transcriptional target of NF-kappaB that blocks TNFalpha-induced apoptosis. *Genes Dev, 13*(4), 382-387.

Zorn, A. M., & Wells, J. M. (2009). Vertebrate endoderm development and organ formation. *Annu Rev Cell Dev Biol, 25*, 221-251. doi: 10.1146/annurev.cellbio.042308.113344

CURRICULUM VITAE

Hyojin Kim

EDUCATION

Doctor of Philosophy, Biochemistry and Molecular Biology 2015

Indiana University

Bachelor of Science, Genetic Engineering 2006

Sung Kyun Kwan University

PROFESSIONAL EXPERIENCE

Ph.D. (2008-2015)

Indiana University, School of Medicine, Department of Biochemistry and Molecular Biology

- Enhanced cell survival and vessel forming ability of endothelial colony forming cell (ECFC) by using human platelet lysates (HPL) and Caspase inhibitors in a three dimensional (3D) collagen matrix model of vasculogenesis *in vivo* and *in vitro*. Cell survival rates and vessel forming ability were analyzed by using multi-color flow cytometry and additional molecular analysis techniques.
- Improved cell survival and vessel forming ability of ECFC by activating Notch signaling with Dll1 stromal cells in a 3D collagen matrix model of vasculogenesis *in vivo* and *in vitro*. Multi-color flow cytometry and

additional molecular analysis techniques were used to analyze cell survival rates and vasculogenesis.

- Developed methods to identify and isolate highly proliferative ECFC using multi-color flow cytometry, multispectral imaging flow cytometry, confocal microscopy and additional molecular analysis techniques.
- Published two manuscripts and two scientific abstracts as a first author.
- Under revision of one manuscript and process in writing one manuscript for submission in 2015

Research Assistant (2007-2008)

University of Utah, School of Medicine, Division of Hematology

- Cloned Guinea pig and Octodon degus Epo and EpoR genes and compared to human Epo and EpoR genes to compare and demonstrate the animals' genetic adaptability of the two species to altitude through high oxygen affinity in blood. Analyzed the molecular mechanism of Hypoxia Responsive Elements (HREs) in mouse Epo genome from various organs by chromatin immunoprecipitation assay (ChIP) to demonstrate the regulatory elements of *EPO* gene response to hypoxic condition in various organs.
- Demonstrated accelerated differentiation of erythropoiesis and gene expressions in polycythemia vera (PV) by molecular analysis of PV in *in vitro* differentiated erythroid progenitors.

- Published two scientific abstracts for conference presentations as a contributing author.

Research Assistant (2004-2005)

Nation Institute of Agricultural Biotechnology, Department of Microbial Genetics

- Developed an isolation method and functional assay to define mechanisms of drug candidates, antifungal materials, from Enterobacter sp. B54 to demonstrate antifungal activities against to Phytophthora Capsici.

Research Assistant (2003-2004)

Sung Kyun Kwan University, Department of Genetic Engineering

- Cloned regulatory genes and downstream genes of the Quorum sensing region of Bacillus subtills genome to characterize Quorum-sensing pheromones signal molecules secreted from Gram-positive bacteria.

PUBLICATIONS AND PRESENTATIONS

Peer-Reviewed

- **Hyojin Kim**, Lan Huang, Paul J. Critser, Zhenyun Yang, Rebecca J. Chan, Lin Wang, Nadia Carlesso, Sherry L. Voytik-Harbin, Irwin D. Bernstein, and Mervin C. Yoder, “Notch ligand Delta-like 1 (Dll1) promotes *in vivo* vasculogenesis in human cord blood derived endothelial colony forming cells (ECFCs)”, Cytotherapy, 2015 pii: S1465-3249 (14) 00883-4.

- **Hyojin Kim**, Nutan Prasain, Sasidhar Vemula, Michael J. Ferkowicz, Momoko Yoshimoto, Sherry L. Voytik-Harbin, and Mervin C. Yoder, “Human platelet lysate improves human cord blood derived ECFC survival and vasculogenesis in three dimensional (3D) collagen matrices”, Microvascular research, 2015 pii: S0026-2862(15)00064-3.

Under-Revision

- Garbacea I, O’Reilly M, Ionescu L, Alphonse RS, Shelley WC, **Hyojin Kim**, Rajabali S, Yoder MC, Thebaud B., “Functional differences between placental micro- and macro-vascular endothelial colony forming cells”, Placenta.

In preparation

- **Hyojin Kim**, Nutan Prasain, Sherry L. Voytik-Harbin, and Mervin C. Yoder, “Inhibition of apoptosis of ECFC enhances human cord blood derived ECFC survival and vasculogenesis in three dimensional (3D) matrices”.

Conference Presentations

- **Hyojin Kim**, Nutan Prasain, Sasidhar Vemula and Mervin C. Yoder, “Methods to improve ECFC survival in 3D collagen gels *in vitro* and promotion of ECFC vascularization *in vivo*”, Abstract of NAVBO Vascular Biology meeting 2014. Abstract and Poster presentation P04.
- **Hyojin Kim**, Ben Wilson, Lan Huang, Irwin Berstein, Lin Wang, Nadia Carlesso and Mervin C. Yoder, “Notch signaling enhances *in vivo* vasculogenic function of human cord blood endothelial colony forming

cells", Abstract of NAVBO Vascular Matrix Biology and Bioengineering Workshop 2011 Vascular Networks program, Abstract and Poster presentation 053-013.

- Donghoon Yoon, PhD, Jihyun Song, MS, **Hyojin Kim**, Bumjun Kim, PhD, Hyejin Shin, MS, Gregory Arnold, PhD, Steven Elliott, PhD, and Josef T. Prchal, MD, "Tissue-Specific HIF-1 and HIF-2 Regulatory Elements and Their Roles in Erythropoietin Gene Expression", Blood, 2011, 112:1303.
- Hana Bruchova, PhD, Donghoon Yoon, PhD, Archana Agarwal, MD, Eva Otahalova, **Hyojin Kim** and Josef T. Prchal, MD, "Erythropoiesis in Polycythemia Vera Is Hyper-proliferative and Has Accelerated Differentiation during *in vitro* Erythroid Expansion and Is Associated with Higher Expressions of *cMYB* and *EPOR* at Early Erythroid Progenitors", Blood, 2007, 110: Abstract 1549.

TECHNICAL SKILLS

Cell biology

Mammalian (human) cell/tissue culture, Immunohistochemistry, Frozen tissue sectioning, Tissue homogenization and cell extraction, Fluorescent microscopy and confocal microscopy, Multi-color (>5) flow cytometry and Multi-color (>5) imaging flow cytometry (Amnis), Annexin V/PI apoptosis assay and Caspase-1 and -3/7 assay, MTT assay, Mouse tissue extraction, *in vitro and in vivo* hypoxia treatment of Guinea pig, Implantation of human

endothelial cell-seeded collagen constructs into NOD/SCID mice, Chromatin immunoprecipitation assay (ChIP)

Computer

FlowJo, Cell Quest, ImageJ, Macintosh and Windows operating systems, Microsoft Office, Photoshop

VOLUNTEER EXPERIENCE

- Trained and managed projects for summer/intern students (undergraduate students and medical students) 2013-2015
- Volunteer to lead and answer questions for Indiana high school students and teachers at Molecular Medicine in Action (MMIA) 2012
- Volunteer to help registration and technical support at US-Korea Conference (UKC) 2011
- Manager of the student's association in Sung Kyun Kwan University